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# Regulatory intricacies that confer temporal control of conjugation and antibiotic production functions in *Streptomyces*: new variations on old themes

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REGULATORY INTRICACIES THAT CONFER TEMPORAL CONTROL OF  
CONJUGATION AND ANTIBIOTIC PRODUCTION FUNCTIONS IN *STREPTOMYCES*:  
NEW VARIATIONS ON OLD THEMES

A Dissertation

Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy

in

The Department of Biological Sciences

by  
Kevin Lee Schully  
B.S., Louisiana State University, 1999  
August 2005

## **DEDICATION**

This work is dedicated to my parents, Lloyd and Sylvia Schully, whose unconditional love and unwavering support throughout my rather lengthy scholastic career made this possible.

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## ABSTRACT

The complex life style of streptomycetes requires that the appropriate program of genes be expressed at the appropriate times. For example, conjugation occurs solely within the substrate mycelium stage of development while antibiotic production occurs in the aerial mycelium. Thus, complex regulatory mechanisms involving numerous modes of control have evolved that confer temporal regulation of functions governing these expression programs. Conjugation of the *Streptomyces lividans* plasmid pIJ101 utilizes only seven plasmid functions in its transmission. Two of these, *tra* and *clt*, are essential while three additional functions, *spdA*, *spdB* and *kilB* augment the process, with KorA and KorB regulating the transfer and spread functions. The transmission operon of pIJ101 is unique, in that multiple layers of transcriptional and posttranscriptional control converging to implement tight control of the transfer and spread functions, specifically the potentially lethal *kilB* gene product. When expressed unregulated, as a chromosomally integrated gene, *kilB* transcription decreases during the later stages of development, in sharp contrast to the temporally increasing pattern of KilB protein. However, when expressed on pIJ101, the *kilB* promoter is largely, if not completely repressed, and expression of *kilB* requires transcription readthrough from upstream. Furthermore, readthrough transcription terminates within a 105 base-pair intergenic region prior to *kilB*. Interestingly, *kilB*-operator-bound KorB repressor appears to act, at least in part, as an attenuator of operon transcription, perhaps by physically barring transcription elongation. Finally, the formation of a stem-and-loop in the readthrough transcript within the intercistronic region appears to promote antitermination of operon transcription to counteract the effects of the KorB roadblock. Regulation of antibiotic production in *Streptomyces* typically involves the streptomycete global regulatory mechanism *bldA*. The sweet potato pathogen *Streptomyces ipomoeae* strain 91-03



produces a bacteriocin-like antibiotic, ipomicin. In liquid culture, ipomicin is produced in low concentrations throughout exponential phase followed by a dramatic 10-fold increase as the culture enters stationary phase. In contrast, transcription of the ipomicin structural gene *ipoA* decreases as the culture ages. The contrasting patterns of *ipoA* transcription and ipomicin production, coupled to the fact that *ipoA* contains a TTA codon in its leader sequence, makes ipomicin a strong candidate for *bldA* regulation.

**CHAPTER ONE:**  
**INTRODUCTION**

The modern proliferation of complete genome sequences has allowed for an assessment of horizontal gene transfer within and between all three domains of life. The information garnered by this research has established horizontal gene transfer as a driving force behind the evolution of species. Analysis of the complete sequence of the *Escherichia coli* genome for example provides for a conservative estimate that approximately 20% of the sequence was obtained horizontally since it diverged from *Salmonella enterica* approximately 100 million years ago (Lawrence and Ochman 1998). Similarly, the recent sequence of the 8.6-megabase-pair (Mb) linear chromosome of the model streptomycete *Streptomyces coelicolor* A3(2) has been divided into three segments: a 4.9 Mb core which is highly related to other actinobacterial genera (*Mycobacterium* and *Corynebacterium*), and two arms which comprise 3.8 Mb of horizontally acquired sequence (Bentley et al. 2002). In both cases, the recently acquired sequences confer the species-specific characteristics, and both organisms are extremely proficient at conjugation.

Horizontal transfer of large genetic elements termed genomic islands has been associated with rapid evolution of bacterial traits. Fitness islands confer upon their recipients the ability to inhabit new environmental niches, allowing them to exist as saprophytes for example (Hacker and Carniel 2001). Similarly, symbiosis islands can allow an organism to positively interact with a new host, such as the genes required for nitrogen fixation contained on the symbiosis islands of *Rhizobium* species (Sullivan and Ronson 1998). Additionally, the acquisition of pathogenicity islands (PAI) is held responsible for the evolution of innocuous bacteria into pathogens (reviewed in Hacker and Kaper 2000, Schmidt and Hensel 2004). Organisms that have long been associated

with the human body as harmless commensals are being converted to aggressive, multi drug resistant pathogens by horizontally acquired sequences (Gill et al. 2005). Mobile genetic elements such as plasmids, transposons, integrons or super-integrons can accumulate antibiotic resistance markers, resulting in antibiotic resistance islands; these can then be transferred in their entirety, producing organisms that are resistant to numerous antibiotics (reviewed in Dobrindt et al. 2004). Also, because gene clusters necessary for the production of antibiotics typically include genes that confer resistance to the effects of those antibiotics, it has been suggested that much of the acquired resistance comes either directly or indirectly from the very antibiotic producing organisms themselves (Benveniste and Davies 1973, Davies 1994, Grohmann et al. 2003).

Horizontal gene transfer has had a major economic impact on the agricultural industry by providing numerous bacteria with the pathogenicity determinants necessary to infect an array of plants. Pathogenicity islands of species of the Gram-negative genera *Erwinia*, *Pseudomonas*, *Xanthomonas* and *Xyella* produce necrotic lesions on the leaves and fruit of numerous economically important crops such as apples, peppers, tomatoes and citrus plants (reviewed in Galan and Collmer 1999, Hueck 1998). Also, a recent surge in research into Gram-positive plant pathogens has identified horizontally acquired pathogenicity islands in the Gram-positive potato pathogens *Streptomyces scabies*, *Streptomyces acidiscabies* and *Streptomyces turgidiscabies* (Kers et al. 2005). Furthermore, with the advent of transgenic plants concern has arisen about the fate of recombinant DNA introduced into the environment by decaying transgenic plants or into the gut by ingested ones. While the legitimacy of this concern has been called into

question (Davidson 2004) and the means to monitor this in nature appear to be lacking (Heinemann and Traavik 2004, Nielsen and Townsend 2004), it seems plausible that naturally transformable bacteria that inhabit the soil or gut could take up and disseminate genetically engineered genes. In fact, it has recently been demonstrated that species of the Gram-negative bacterium *Acinetobacter* were capable of taking up and incorporating into its genome the DNA of a transgenic tobacco plant in soil (de Vries et al. 2004). Could genetically engineered DNA from the soil be taken up by naturally transformable microbes and disseminated by a horizontal gene transfer mechanism such as conjugation?

Conjugation among Gram-negative bacteria is exemplified by the fertility factor F of *E. coli*. At 100 kb in size, F encodes all of the functions (approximately 35 gene products expressed from 1/3 of the entire plasmid) involved in the processing and transmission of F DNA (Bushman 2002). Additionally, F is capable of integrating into at least 20 sites on the *E. coli* chromosome at which time it can promote the transfer of up to one unit length of the *E. coli* genome (Reimann et al. 1993). Conjugal transfer of F begins with the plasmid-encoded sex pilus, which extends from the donor, attaching to the recipient. The pilus then retracts until intimate contact is made between the donor and the recipient and the mating pore is formed (Durrenberger et al. 1991). Processing of F DNA occurs prior to mating and is initiated with a site-specific nick at the origin of transfer (*oriT*) by the plasmid-encoded endonuclease relaxase. Additional proteins complexed with relaxase form the relaxosome and the 5' end of F becomes bound to the mating pore. Plasmid DNA is then transferred, 5' to 3', into the recipient cell. In the donor, rolling circle replication (RCR) reconstitutes the parental plasmid probably by continuous DNA synthesis initiated from the free 3' hydroxyl generated during strand

nicking. In the recipient, the new plasmid is completed using the new host's DNA replication machinery (Lanka and Wilkins 1995).

The genus *Streptomyces* is comprised of Gram-positive, spore-forming, predominately soil-dwelling bacteria and is known for a number of hallmark features that distinguish its members from the majority of other prokaryotic genera. Perhaps the most notable feature of streptomycetes is a period of physiological differentiation known as secondary metabolism wherein many species of the genus *Streptomyces* are renowned for their production of thousands of important compounds including approximately 80% of the 10,000 naturally produced antibiotics (Kieser et al. 2000). Also, contrary to the majority of bacteria, streptomycetes undergo a complex life cycle that includes three morphologically distinct stages (Chater 1993). Finally, members of the genus *Streptomyces* have been known to possess an extremely efficient, although poorly characterized, system of conjugal gene transfer that may exemplify a novel mechanism for conjugation (Reviewed in Grohmann et al. 2003).

An overwhelming majority of the research conducted on *Streptomyces* bacteria has been dedicated to the production of secondary metabolites for their medical and industrial importance and their economic return. As a result, basic research type endeavors have received little attention, leaving the understanding of mechanisms such as conjugation among *Streptomyces* spp. lagging far behind their Gram-negative counterparts. Mounting evidence however presented by a few investigators implicates a novel mechanism governing streptomycete conjugation. For example, in contrast to the single-strand transmission observed in all other bacteria to date, Possoz et al. (2001) recently demonstrated that transfer of the *S. ambifaciens* plasmid pSAM2 between

*Streptomyces* strains probably occurs as a double-stranded molecule. Here, the authors utilized the type-two restriction modification system *SalI* RM, which was either present or absent in the recipient strain and absent in the donor strain. When the recipient lacked the *SalI* RM system, conjugal transfer of pSAM2 occurred with high efficiency.

However when the recipient strain contained the *SalI* RM system, restriction by the recipient all but abolished transfer of pSAM2. Since single-stranded DNA is resistant to such restriction, this result suggested that transfer among the *Streptomyces* species most probably occurs by an as-yet-unknown double-stranded mechanism. In addition to this evidence, the apparent lack of a sex pilus, lack of detectable pheromones and remarkably few plasmid-encoded functions all combine to implicate a potentially novel mechanism.

Conjugation in *Streptomyces* is believed to occur in two phases: intermycelial transfer in which plasmids are transferred from the donor to the recipient and intramycelial transfer in which the plasmids are disseminated throughout the recipient hyphae in a process termed “spread” (Hopwood and Kieser 1993, Kieser et al. 1982). Plasmid transmission may be observed macroscopically in *Streptomyces* species through the formation of “pocks”. This phenomenon is distinctive to streptomycete conjugation and is associated with the unique filamentous growth demonstrated by these bacteria (Bibb et al. 1981, Hopwood and Keiser 1993). When a *Streptomyces* spore germinates, growth occurs by apical extension and branching forming hyphae that are separated into multigenomic compartments by infrequently spaced septae. The result is a matted mass of interwoven mycelia that grow along the surface of the substrate and penetrate the substratum. It is during this period of development that *Streptomyces* bacteria are capable of efficient conjugal transfer of their genetic complement (Hopwood and Kieser

1993, Pettis and Cohen 1996). As nutrients become exhausted, this mode of growth ceases and gives rise to the aerial mycelium. Individual hyphae erupt from the substrate and growth is directed vertically. These aerial hyphae are also infrequently septated, and it is during this stage of development that secondary metabolism commences. In preparation for sporulation, the aerial hyphae coil, become frequently septated producing compartments containing a single copy of the genome and finally differentiate into dispersible spores (Chater 1993). Submerged *Streptomyces* cultures grow as small bundles of mycelia that are visible to the naked eye, and the cultures follow the typical bacterial growth curve. Submerged mycelia do not differentiate morphologically nor do they conjugate; they do, however, undergo secondary metabolism as the culture enters stationary phase (Champness and Chater 1994). When a spore containing a conjugal plasmid germinates within a lawn of substrate mycelia, pocks, or circular zones of retarded growth (i.e., delayed development of aerial hyphae) appear. All of the mycelia within the pock have received a copy of the plasmid (Bibb and Hopwood 1981).

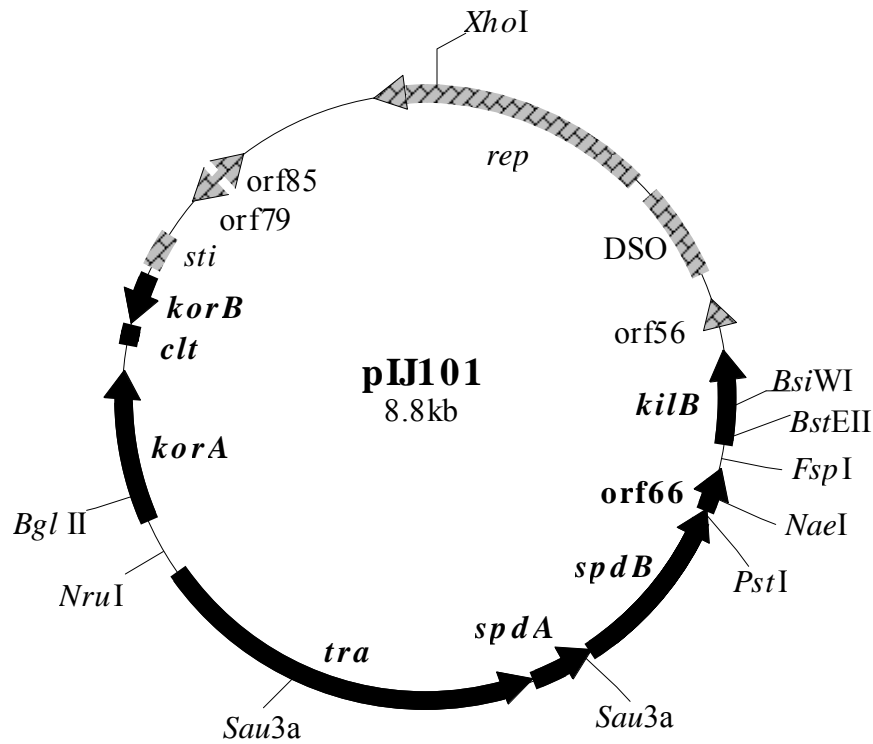
One of the most extensively studied plasmids of streptomycetes, pIJ101 (Figure 1.1), is an 8.8-kilobase, circular, autonomously replicating, high-copy plasmid isolated from *Streptomyces lividans* (Kieser et al. 1982) that is capable of transferring itself to up to 100% of all potential recipients as well as promoting the transfer of chromosomal markers, yielding up to 1% recombinants. Genetic analysis of pIJ101 has indicated that seven plasmid loci are involved in the transmission of pIJ101 and only two of these loci are required for plasmid transfer. One of these is *tra* (Figure 1.1) (Kendall and Cohen 1987), which encodes a 70-kDa membrane protein expressed exclusively in the substrate mycelium (Pettis and Cohen 1996) and which interestingly, bears significant homology to



proteins from other bacteria that are known to function in the intracellular transport of double stranded DNA (Kendall and Cohen 1988, Kosono et al. 1996). Additionally, *clt* (Figure 1.1), a 54 base pair *cis*-acting locus of transfer (Pettis and Cohen 1994, Ducote et al. 2000) is also required in *cis* for transmission of pIJ101. Three other plasmid functions, *spdA*, *spdB* and *kilB* (Figure 1.1) make the conjugation process more efficient but are not required and thus apparently augment plasmid transfer through the aforementioned spread function (Kendall and Cohen 1987). Two remaining plasmid-encoded proteins participate in the regulation of the transfer and spread functions. KorA is a negative transcriptional regulator of the *tra* promoter as well as of its own promoter, and KorB represses transcription from the *kilB* promoter as well as the *korB* promoter (Kendall and Cohen 1987). *tra*, *spdA*, *spdB* and *orf66*, a small open reading frame of unknown function, comprise the transmission operon and are expressed as a single polycistronic message, which originates from a promoter upstream of the *tra* ORF but which lacks a traditional termination sequence downstream of *orf66* (Kendall and Cohen 1988). *kilB* is expressed in the same orientation as the transmission operon but is separated from the upstream genes by a 105-bp intergenic region, which contains the *kilB* promoter (Kendall and Cohen 1988, Stein et al. 1989).

KilB was first implicated as a spread protein following a pUC19 insertion into the *kilB* ORF that resulted in a 4-fold reduction in pock size (Kendall and Cohen 1987). The KilB protein is approximately 15-kDa, very hydrophobic, and so far appears unrelated to any other proteins (Kendall and Cohen 1988). KilB is lethal to the cell when the *kilB* gene is expressed in high copy in the absence of KorB (*kor* designates kil-override), but the efficiency of plasmid transmission is significantly reduced in its absence (Kendall and

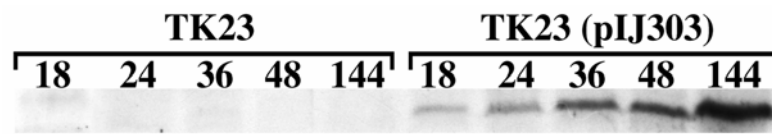
Cohen 1987). KorB protein is a small transcriptional repressor, which suppresses the KilB-associated lethality of *kilB* overexpression (Kendall and Cohen 1987, Stein et al. 1989). KorB is expressed as a 10-kDa protein that is processed into a 6-kDa functional form (Tai and Cohen 1993), which binds to the *kilB* promoter from -40 to +21 relative to the *kilB* transcription start site (Ducote and Pettis 2003), thereby implicating a mechanism where several molecules of KorB are bound to the operator sequence.



**Figure 1.1. Genetic organization and physical map of pIJ101.** The plasmid has been divided into two segments: the top section indicated here by the pattern-filled arrows and boxes contains the replication and stability region and the lower section highlighted by the filled arrows and boxes indicate those plasmid loci involved in plasmid transmission. Several restrictions sites that are relevant to this work are also indicated. (Reproduced from Pettis et al. 2001).

In cultures of *S. lividans* strain TK23 harboring pIJ303, a fully functional thiostrepton-resistant derivative of pIJ101 (Kieser et al. 1982), the concentration of KilB protein increases temporally throughout *Streptomyces* growth. This temporal increase in expression or accumulation occurs throughout cellular differentiation when mycelia are grown on solid media (Figure 1.2 A), such that KilB is found at its lowest concentration in the substrate mycelium (Figure 1.2 A, 18hrs). Steady state concentrations of KilB protein then progressively increase so that it is found at its highest concentration in the spores, approximately 13-fold greater than in the substrate (Figure 1.2 A, 144 hrs). This temporal increase can also be observed when *S. lividans* harboring pIJ303 is grown submerged in liquid culture (data not shown), suggesting that the temporal increase in KilB protein expression or accumulation is not related to morphological cues that regulate cellular differentiation (Pettis et al. 2001).

A.



B.



**Figure 1.2. Western blot analysis of KilB protein throughout cellular differentiation.** Spores of (A) TK23 or TK23 harboring pIJ303, a thiostrepton derivative of pIJ101, or (B) *S. lividans* strain NWR2 were grown on solid media through one complete life cycle. Mycelia were harvested at the timepoints indicated (in hours) and used to make total protein extracts. Equivalent amounts of the resulting extracts were electrophoresed by PAGE and analyzed by Western blot. (Reproduced from Pettis et al. 2001.)

Because the copy number of pIJ101 increases throughout the streptomycete life cycle (Keiser et al. 1982), it was possible that the observed increase in KilB concentration was simply a function of more copies of the *kilB* gene at later stages of growth. It was also possible that variations in KorB repression of the *kilB* promoter were responsible for the temporally increasing concentration of KilB protein. With *S. lividans* strain NWR2 (Pettis et al. 2001) it was possible to simultaneously address these two questions. Strain NWR2 contains a single unregulated copy of the *kilB* gene integrated into the *S. lividans* chromosome, guaranteeing that the copy number remains invariant throughout the life cycle. Additionally, the lethal effects of unregulated *kilB* expression were apparently circumvented by the low *kilB* gene dosage. *S. lividans* strain NWR2 produced a similar, though elevated trend of temporally increasing KilB on solid media (Figure 1.2 B) as well as in submerged culture (data not shown) (Pettis et al. 2001). It is interesting to note the elevated levels of KilB observed in strain NWR2. Comparing the concentration of KilB protein in NWR2 at 144 hrs to TK23(pIJ303) at the same timepoint (Figure 1.2 B), especially when an approximate 100-fold difference in copy is considered, these data suggest that substantial emphasis is placed on the regulation of this lethal protein.

Although the previously mentioned hallmark features separate *Streptomyces* spp. from other prokaryotes and are assumed to be ubiquitous throughout the genus, there are some species that distinguish themselves with unique features within the streptomycetes. Pathogenicity for example appears to be a rare occurrence among *Streptomyces* species and with one exception (*S. somaliensis*) those pathogenic species are plant pathogens (Kieser et al. 2000). Like conjugation, research dedicated to streptomycete pathogens has been largely overlooked. However, as both the list of plant pathogenic streptomycetes

and their economic impact on the crops they infect increases (reviewed in Loria et al. 2003 and referenced therein), more and more researchers are focusing their efforts on pathogenic streptomycetes. Research in this area has determined that analogous to the streptomycete conjugation paradigm, *Streptomyces* plant pathogens possess novel microbial virulence properties. Unlike the majority of plant pathogenic bacteria, *Streptomyces* species are capable of infecting roots, as opposed to aerial plant tissue. This occurrence is believed to be facilitated by the production of unique compounds called thaxtomins, nitrated dipeptide phytotoxins (Lawrence et al. 1990, Healy et al. 2000) that inhibit cellulose biosynthesis and aid in plant cell invasion.

The first and most thoroughly investigated plant pathogenic species, *S. scabies* (Lambert and Loria 1989 a), causes the common potato scab, and it is the most economically important streptomycete pathogen. *S. scabies* is distributed worldwide and produces erumpent scab disease characterized by raised, rough and corky lesions in the storage root of potato and other root crops such as turnip, carrot, radish and peanut (Loria et al. 1997). Genetically distinct but symptomatically indistinguishable from *S. scabies* are two additional plant pathogenic streptomycetes: *S. acidiscabies* (Lambert and Loria 1989 b), which is capable of infecting the same crops as *S. scabies*, but at much lower (4.2) soil pH (Loria et al. 1997), and *S. turgidiscabies*, which was recently isolated in Japan (Miyajima et al. 1998). An array of thaxtomins is produced in each of these species but thaxtomin A is their major virulence factor (Loria et al. 1997). Following thaxtomin-facilitated invasion, the *necI* gene product induces necrosis in the plant tissue (Bukhalid and Loria 1997). All of the functions required to produce disease symptoms are present on a large pathogenicity island (Kers et al. 2005), the first discovered in a

Gram-positive plant pathogen, that has been demonstrated to be horizontally transferable (Bukhalid and Loria 1997). Additionally, evidence suggests a cascade of recent horizontal transfer events occurred in which pathogenicity was first acquired by *S. scabies* from an unrelated taxon and was subsequently passed to *S. acidiscabies* and *S. turgidiscabies* (Bukhalid and Loria 1997, Healy et al. 1999).

*Streptomyces ipomoeae* is a pathogen of the sweet potato *Ipomoea batatas* (Clark and Moyer 1988). Termed “soil rot”, *S. ipomoeae* produces necrotic lesions on the storage root, thereby reducing its market value, as well as destroying the feeder roots, thereby reducing storage root yields (Clark and Matthews 1987). Thus, *S. ipomoeae* has had a major economic impact on the sweet potato industry. Besides disease symptomology, *S. ipomoeae* differs from the common scab-causing *Streptomyces* species in that it is not known to infect potato, it produces thaxtomin C as its major virulence factor (King et al. 1994) and it lacks the *nec1* gene (Bukhalid and Loria 1997).

Because the soil is not a nutrient-rich environment, it is necessary for the inhabitants to compete for available nutrients. This competition is believed to be the driving force behind the evolution of bacterial antagonism and the production of so many compounds vital to industry, medicine and agriculture for which *Streptomyces* species are renowned. In addition to broad spectrum antibiotics with the potential to affect the growth of many different organisms, inhibitory compounds with a much narrower effective range are also produced. One class of proteinacious antimicrobial agents termed bacteriocins are typically capable of inhibiting only those organisms that are very closely related to the producers (Jack et al. 1995).

*S. ipomoeae* is comprised of many strains that can be divided into three groups based on their ability or inability to antagonize members of the other groups. Members of group I do not produce an antagonist and their growth is inhibited by products of groups II and III. *S. ipomoeae* group II strains produce a colony-associated inhibitor of groups I and III while strains of group III produce a diffusible inhibitory compound of Groups I and II (Clark et al. 1998). The group III inhibitor has been purified and determined to be a 10-kDa bacteriocin-like protein designated ipomicin, only the second identified bacteriocin-like protein produced by a streptomycete (Roelants and Naudts 1964). The N-terminus of ipomicin was sequenced and used to identify its structural gene, *ipoA*, on the *S. ipomoeae* chromosome, where it contains additional sequence on the 5' end; this result implies that ipomicin is expressed as a 13-kDa precursor, which is processed into the 10-kDa form that is secreted into the medium (Zhang et al. 2003).

In this work, I will address two areas of *Streptomyces* research that have received less attention – conjugation as exemplified by plasmid pIJ101 and biology of a plant pathogenic streptomycete, *S. ipomoeae*. In chapters two and three, I will describe the unique transmission operon of pIJ101, which I show includes not only *tra*, *spdA*, *spdB* and *orf66* but also *kilB*, and which demonstrates unprecedented control upon the expression of the distal gene (*kilB*). Control of this operon involves a previously unknown mode of transcription termination, which results in attenuation of the operon transcript, as well as antitermination, which acts in opposition to the terminator to affect the tightly controlled expression of *kilB*. The resulting multipartite transcriptional regulation links the expression of *kilB* to the transmission operon, placing *kilB* primarily under the control not of the *kilB* promoter regulated by the KorB repressor, but of the *tra*

promoter and the previously unknown terminator/antiterminator system. Additionally, in chapter four, I will describe the expression characteristics of the *S. ipomoeae* group III inhibitor, ipomicin, and provide preliminary evidence for its regulation by the *Streptomyces* global regulator *bldA*.

## References

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## **CHAPTER TWO:**

### **SEPARATE AND COORDINATE TRANSCRIPTIONAL CONTROL MECHANISMS LINK EXPRESSION OF THE POTENTIALLY LETHAL *kilB* SPREAD LOCUS TO THE UPSTREAM TRANSMISSION OPERON ON *STREPTOMYCES* PLASMID pIJ101\***

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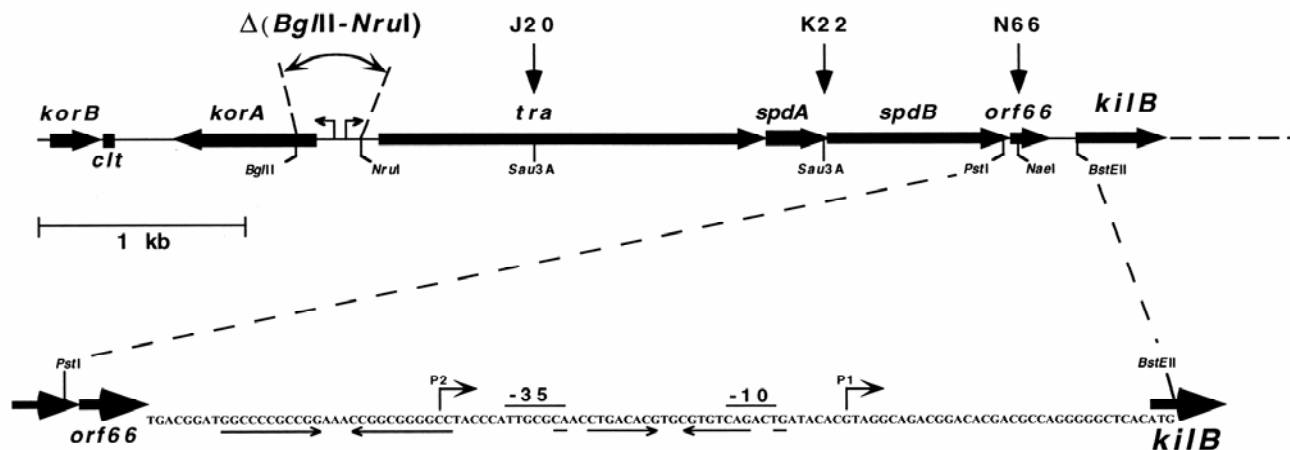
## Introduction

*Streptomyces* are Gram-positive, predominantly soil bacteria that display a complex life cycle involving three morphologically distinct stages and the production of an array of important chemicals during a period of secondary metabolism. In the initial substrate mycelium stage that is seen during surface growth, streptomycetes grow as a matted mass of interwoven mycelia that are separated into multigenomic compartments by infrequently spaced septae (Chater 1993, Chater 1998). It is also exclusively during this period of development that streptomycete bacteria are capable of conjugal transfer (Pettis and Cohen 1996). As nutrients become exhausted, growth is directed vertically in the form of aerial hyphae and secondary metabolism commences. Destined to differentiate into dispersible spores, individual aerial hyphae coil and become frequently septated, thereby producing compartments containing a single copy of the genome (Chater 1993, Chater 1998). Throughout typical exponential and stationary growth phases in laboratory broth cultures, species such as *S. lividans* grow undifferentiated as mycelia with secondary metabolism occurring as the submerged cultures enter stationary phase (Champness and Chater 1994).

Genetic exchange among streptomycete bacteria, though poorly understood, is well documented to occur at high frequency and seems to require the presence of plasmids (Hopwood and Kieser 1993). One plasmid, pIJ101, is an 8.8 kb, high copy plasmid that is capable of transferring itself with up to 100% efficiency among *Streptomyces* species and has a broad host range among actinomycete bacteria. In addition, pIJ101 is capable of promoting the transfer of the host chromosome while existing exclusively as an autonomous circular DNA molecule (Kieser et al. 1982).

Genetic analysis has identified five loci that are directly involved in the transmission of pIJ101 and has further shown that only two of these are necessary and sufficient for intermycelial transfer. These latter two loci consist of *clt*, a *cis*-acting transfer sequence (Ducote et al. 2000, Pettis and Cohen 1994), and *tra* (Kendall and Cohen 1987) (Figure 2.1), which encodes a 70-kDa protein whose temporal expression exclusively within the substrate mycelium coincides with the timing of pIJ101 transfer (Pettis and Cohen 1996). The remaining three loci, *spdA*, *spdB* and *kilB* (Figure 2.1) appear to augment plasmid transfer (in a related process termed “spread”) as mutations in their coding sequences reduce but do not eliminate the lateral transmission of plasmids from donor to excess recipient mycelia during matings on agar surfaces (Kendall and Cohen 1987). The spread genes may contribute to overall plasmid transmission by promoting intramycelial movement of plasmids throughout recipient mycelia (Hopwood and Kieser 1993, Pettis et al. 2001).

The genes *tra*, *spdA* and *spdB* appear to be encoded on a constitutively expressed polycistronic message (Pettis and Cohen 1996) that originates upstream of the *tra* open reading frame (ORF) and includes *orf66* (Figure 2.1), a small ORF of unknown function (Kendall and Cohen 1988). Downstream of *orf66* and the rest of the upstream transmission operon exists the *kilB* gene, whose expression has been considered to be controlled from two distinct putative proximal promoters (Stein et al. 1989), which for convenience will be referred to here as P1 and P2. The initiation site for the P1 promoter (labeled “P1” in Figure 2.1) was identified in *S. lividans* using first S1 nuclease mapping (Buttner and Brown 1985) and later primer extension (Stein et al. 1989) and was also shown to be functional in the heterologous host *E. coli* (Buttner and Brown 1987).



**Figure 2.1. Genetic organization and physical map, including the locations of various mutations, of the transfer and spread region of *Streptomyces* plasmid pIJ101.** Open reading frames (ORFs) are shown with the direction of translation (filled arrows) as well as their promoters (small bent arrows on line drawings). The positions of previously described (Kendall and Cohen 1987) pUC19 insertions (i.e., J20 and K22) on the conjugative pIJ101 derivative pIJ303 (Kieser et al. 1982) are indicated as is the location on the conjugative pIJ101 plasmid pHYG3 (Pettis and Cohen 1994) of a kanamycin resistance cassette insertion (N66). The extent of a deletion ( $\Delta BglII-NruI$ ) created on pIJ303 is also shown. Shown enlarged below the entire transfer and spread region is one strand of the 106-bp sequence that lies between the small ORF of undetermined function *orf66* and *kilB*, including the previously described (Stein et al. 1989) major and minor *kilB* transcription start sites (designated here P1 and P2 for their respective promoters) along with putative P1 promoter determinants. Inverted repeats are indicated including one set which overlaps P1 promoter sequences and appears to constitute the KorB operator for this gene (Tai and Cohen 1993, Zaman et al. 1992) as well as another set positioned just downstream from *orf66*.



P1 appears to be the major promoter and has putative –10 and –35 determinants (Figure 2.1) that strongly resemble promoter sequences recognized by *E. coli* RNA polymerase containing  $\sigma^{70}$  (Strohl 1992). Using primer extension, Stein et al. (1989) also saw evidence for an additional transcription initiation site for the *kilB* gene functioning in *S. lividans* (designated “P2” in Figure 2.1), whose putative promoter determinants do not resemble consensus *E. coli* promoter sequences (Strohl 1992).

Two other pIJ101 loci, *korA* and *korB* (Figure 2.1) encode transcriptional repressors, which act on the *tra* promoter and the P1 promoter of *kilB*, respectively, as well as on their own cognate promoters (Stein et al. 1989, Stein and Cohen 1990). The importance of such regulation by the KorA and KorB repressors was underscored by initial results showing that unregulated expression from the *tra* promoter (formerly *kilA*) and of the *kilB* gene are lethal to the host cell when occurring on high-copy pIJ101 replicons (Kendall and Cohen 1987). Recently, further evidence of the deleterious effects of unregulated *kilB* expression was provided by experiments where a single copy of the pIJ101 *kilB* gene including its entire promoter region was inserted into the *S. lividans* chromosome; the resulting strain, NWR2, which produced greatly elevated amounts of KilB protein, became genetically unstable upon repeated passaging and was eventually deleted for its *kilB* chromosomal sequences at a high frequency (Pettis et al. 2001).

We previously began characterization of the pIJ101 spread components by identifying KilB protein in extracts of *S. lividans* cells and demonstrating that KilB temporally increases in concentration both in pIJ101-containing cells as well as in strain NWR2 (Pettis et al. 2001). Because the latter strain lacks the pIJ101 *korB* gene, the temporal increase in KilB protein is known to occur independently of KorB

transcriptional regulation. Also, this pattern of KilB expression or accumulation occurred both in differentiating surface cultures as well as non-differentiating liquid cultures and thus is not linked per se to morphological changes during *Streptomyces* growth (Pettis et al. 2001).

Here in an effort initially to define further the mechanism governing the temporal increase in KilB protein, we used a reporter gene construct, the ribonuclease protection assay (RPA), and primer extension analysis of *kilB* mRNA to show that, in contrast to KilB protein, transcription initiating from the *kilB* promoter region peaks during exponential growth and then decreases at later times. Unexpectedly, these studies provided evidence that the *kilB* gene on pIJ101 is also expressed as part of the polycistronic message that includes the other upstream transfer and spread functions. Subsequent insertion and deletion mutations at positions within the upstream operon were found to reduce or abolish expression of KilB protein, despite the presence of the downstream intercistronic *kilB* promoter region. Although transcription originating from the *kilB* promoter was shown to be dramatically reduced by KorB binding under non-mating conditions, our results are nevertheless consistent with a mechanism involving at least some RNA polymerase readthrough of KorB-bound *kilB* promoter sequences as a means for the coordinated expression of the *kilB* gene with the upstream transmission operon. The possible roles of coordinate and separate transcriptional control mechanisms in regulating expression of the potentially lethal *kilB* function under both mating and non-mating conditions are discussed.

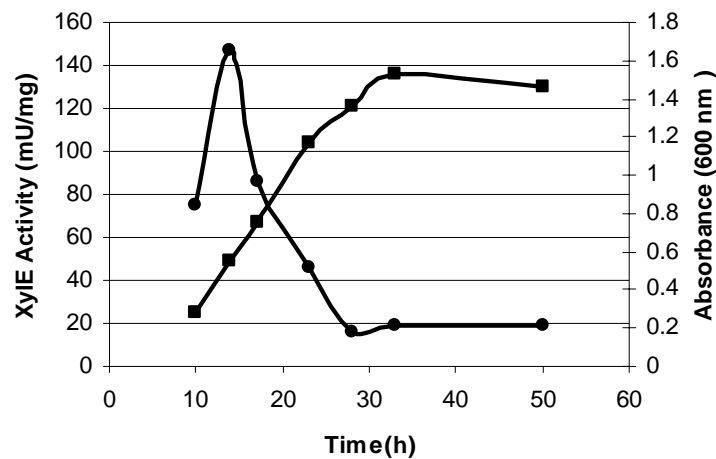
## Results

### **The *kilB* Promoter Region Is Most Active during Exponential Growth**

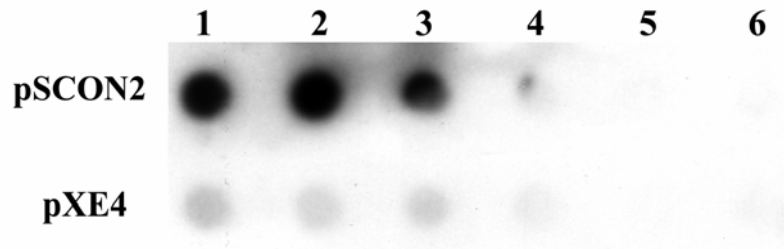
Although transcriptional control by KorB was previously ruled out as a basis for the temporal increase in KilB protein seen during growth of *S. lividans*, it was still possible that some alternative mechanism operating at the level of transcription might be responsible. To determine whether there is a correlation between *kilB* promoter activity and KilB protein concentration during *S. lividans* growth, we utilized plasmid pSCON2 (Ducote and Pettis 2003), a derivative of the promoter probe vector pXE4 (Ingram et al. 1989) in which the 0.4 kb *Bst*EII-*Pst*I fragment containing the *kilB* promoter region (Figure 2.1) was cloned upstream of the promoterless *xylE* gene on this replicon. The *xylE* gene encodes catechol dioxygenase, an enzyme which catalyzes the conversion of catechol to an oxidized derivative that is characterized by an easily assayable yellow color. Upon transformation of *S. lividans* strain TK23 with pSCON2, transformants growing in liquid YEME medium were assayed at increasing culture densities for *kilB*-promoter-driven catechol dioxygenase activity as described in the Materials and Methods section.

The *kilB* promoter region was found to be most active early in exponential growth for pSCON2-containing cells (Figure 2.2 A) as catechol dioxygenase appeared to reach a maximum (147 mU/mg of total protein) by only the second timepoint taken (absorbance at 600 nm = 0.547); after this point, function of the *kilB* promoter showed a relatively rapid and precipitous decline so that by the time cultures were transitioning into stationary phase (i.e., the fifth timepoint, absorbance at 600 nm = 1.357), reporter enzyme activity had dropped to 16 mU/mg of total protein, a level which then remained

**A.**



**B.**



**Figure 2.2. *kilB*-promoter-driven *xylE* gene expression and activity.**

**A)** Growth curve and corresponding catechol dioxygenase activity profile for *S. lividans* strain TK23 harboring pSCON2. Cultures were grown in liquid YEME medium containing thiostrepton until an absorbance at 600 nm of approximately 0.15, whereupon they were diluted into fresh YEME medium (time 0). Aliquots were then subsequently harvested at the indicated times and used either to determine the absorbance of the culture (■) or to prepare extracts for determination of catechol dioxygenase activity (●), which was assayed and expressed in milliunits per mg of total protein all as described in Materials and Methods. The data presented are the results from one experimental trial although six independent trials were conducted with approximately the same results in each case.

**B)** Dot blot for *xylE* message. Total RNA was isolated from additional mycelia harvested at each of the first six timepoints in (A) for TK23(pSCON2) and for similar timepoints for TK23 containing the promoterless vector pXE4, and 5-μg aliquots were affixed (in order in lanes 1-6) to Hybond nylon membrane, and hybridized to a radiolabelled *xylE*-specific DNA probe using conditions described previously (Brasch et al. 1993). Dots were then visualized by autoradiography.

approximately the same throughout the remainder of the assay. In contrast, catechol dioxygenase in similar temporal assays involving TK23 cells harboring the vector pXE4 never exceeded 6.0 mU/mg of total protein (data not shown).

These data were confirmed by probing for *xylE* mRNA in total RNA of TK23(pSCON2) cells isolated at the identical timepoints used for the catechol dioxygenase assays. Using a radiolabelled *xylE*- specific DNA probe, the pattern of *xylE* message seen in dot blots (Figure 2.2 B) followed the above trend in catechol dioxygenase activity closely, with the concentration of *xylE* mRNA originating from the *kilB* promoter increasing from the first to the second timepoint and then rapidly declining to a point where it was no longer detectable by this method. Again, *xylE* mRNA originating from the pXE4 vector alone was negligible.

#### **Temporal Increase in Intercellular KilB Concentration Is Regulated Post-transcriptionally**

The results obtained above using pSCON2 suggested that expression of the *kilB* message itself does not temporally increase throughout *Streptomyces* growth. Since numerous attempts to visualize steady state *kilB* mRNA by Northern blotting were unsuccessful probably owing to instability of this message (data not shown), a RPA specific for the 5' end of the *kilB* message was employed. Here, a radiolabelled riboprobe of 407 nucleotides (nt) (Figure 2.3 A) was used along with RNA isolated at various times (Figure 2.3 B) throughout the exponential and stationary phases of liquid growth of *S. lividans* NWR2, the previously described strain which contains a chromosomal copy of the pIJ101 *kilB* gene including its entire promoter region (Pettis et al. 2001). Based on previous transcriptional mapping studies (Stein et al. 1989), the

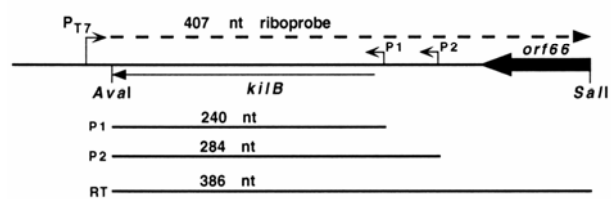
**Figure 2.3. Contrasting profiles of the *kilB* message and KilB protein during growth of *S. lividans* strain NWR2.**

**A)** Synthesis of a *kilB*-specific riboprobe. pSCON33 was linearized at *SalI* within *orf66* and a 407-nt radiolabelled riboprobe was synthesized in vitro in the direction shown using T7 RNA polymerase, [ $\alpha$ - $^{32}$ P]-CTP and remaining cold ribonucleotides. The positions of the previously determined (Stein et al. 1989) transcription start sites within the *kilB* promoter region are indicated. Shown below the physical and genetic map are the extent and size of possible *kilB*-containing transcripts that would hybridize to the probe. RT, readthrough transcription.

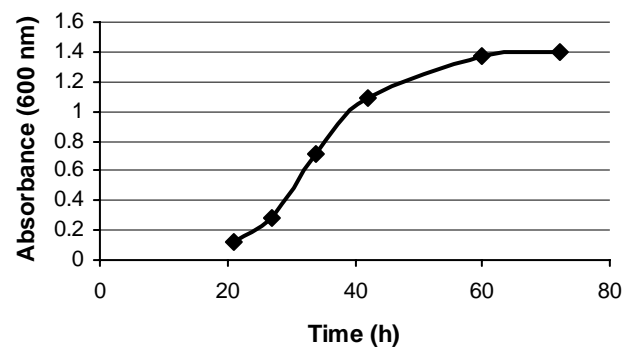
**B)** Growth curve for *S. lividans* NWR2. A culture of strain NWR2 was grown in liquid YEME medium, and samples were harvested at the indicated timepoints 1-5 for measurement of culture absorbance at 600 nm as shown here or for isolation of RNA for part (C) or for preparation of protein extracts for part (D).

**C)** RPA of *kilB* message in strain NWR2. Using total RNA isolated from cells harvested at the timepoints shown in (B) along with the riboprobe described in (A), RPAs were performed as described in Materials and Methods, and reactions were resolved by gel electrophoresis in order in lanes 1-5 alongside a DNA sequencing ladder for size approximation, with subsequent visualization of bands by autoradiography. The reaction in lane 6 involved total RNA isolated from strain TK23 grown to an absorbance at 600 nm of 1.346. Based on comparison to the DNA size ladder, protected RNA fragments of approximately 240 nt (for P1) are indicated as is the expected migration position (indicated by the asterisk) of protected fragments that would be indicative of P2-initiated transcription and the expected position (indicated by the double asterisk) of fragments that would be derived from readthrough transcription. The RPA experiment as presented was performed a total four times, and the highest concentration of P1-derived protected fragment was consistently seen in early to late exponential phase growth with lesser amounts then appearing at later timepoints. (D) Western blot of KilB protein in strain NWR2. Protein extracts from NWR2 cells harvested at the timepoints indicated in (B) were prepared and analyzed in order in lanes 1-5 by Western blotting using a previously described antiserum against KilB (Pettis et al. 2001).

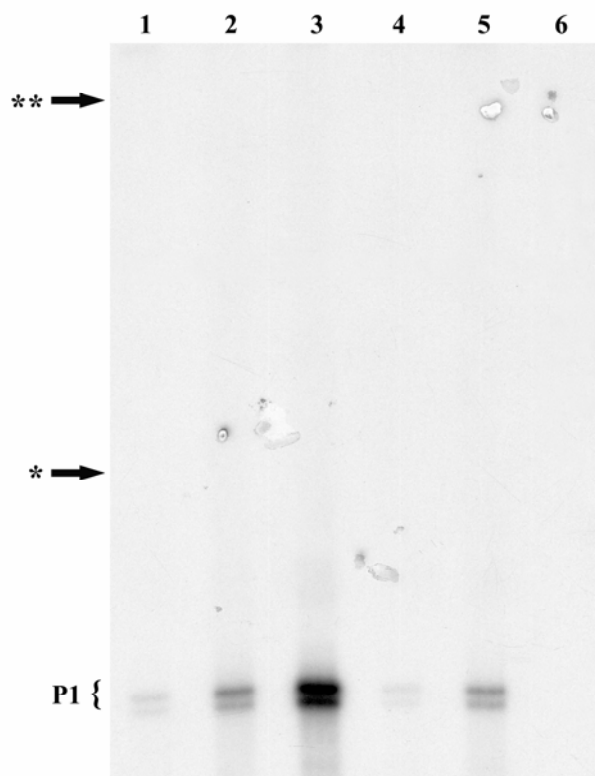
A.



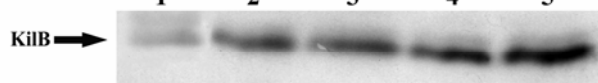
B.



C.



D.



riboprobe would be complementary to the first 240 nt of transcripts that originate from the P1 *kilB* promoter as well as the first 284 nt of transcripts that originate from the P2 promoter (Figure 2.3 A). Transcription originating from upstream of P2, which in the case of strain NWR2 would likely be derived from exogenous host sequences located beyond the chromosomally inserted copy of *kilB*, would be complementary to 386 nt of the riboprobe (Figure 2.3 A, designated RT for “readthrough transcription”).

RPAs performed using NWR2-derived RNA yielded a doublet of protected RNA fragments of the size expected (i.e., ~240 nt) for transcripts originating from the P1 promoter (Figure 2.3 C, lanes 1-5), although the presence of two potential start sites for P1 in *S. lividans* instead of only one differed from previous results (Stein et al. 1989, Buttner and Brown 1985). The doublet seen here was absent in a RPA involving strain TK23 RNA (lane 6) and in reactions using NWR2 RNA that had been pre-treated with RNase prior to subsequent removal of the RNase and completion of the RPA protocol (data not shown). The pattern of protected fragments obtained with RNA from strain NWR2 indicated that the 5' end of P1-initiated *kilB* messages increased in amount throughout exponential phase reaching its highest concentration when cultures were still growing exponentially and was then present in lesser amounts during the subsequent stationary growth period (compare Figure 2.3 B and 2.3 C). No protected fragments of the size expected for P2-derived transcripts were evident for strain NWR2 (their predicted position is marked with an asterisk in Figure 2.3 C) despite the fact that the chromosomally inserted pIJ101 sequence in this strain (Pettis et al. 2001) extends well upstream of the putative P2 promoter position (Stein et al. 1989, Strohl 1992). Similarly,

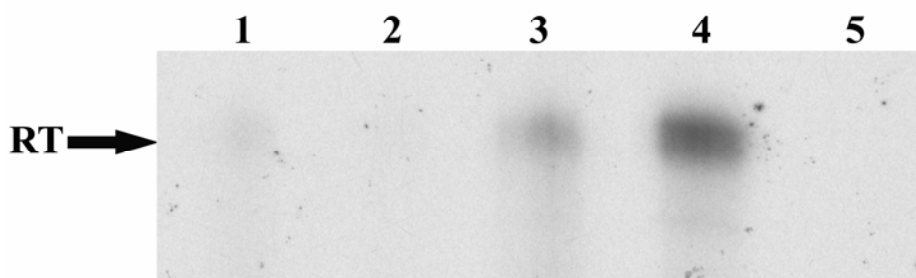


no fragments of the size indicative of readthrough transcription (whose expected position is marked with a double asterisk in Figure 2.3 C) were seen.

The pattern of P1-initiated *kilB* mRNA contrasted significantly with that of KilB protein, which as expected showed a steady temporal increase in concentration in Western blots (Figure 2.3 D) that involved NWR2 cell extracts prepared at the identical timepoints used for the RPA along with previously described antibodies against KilB (Pettis et al. 2001). Since this consistent temporal increase in KilB in NWR2 cells was not accompanied by a concurrent steady increase in *kilB* mRNA, these data indicate the existence of a previously unknown posttranscriptional mechanism for regulating intracellular KilB protein concentration.

#### **The *kilB* Gene Is part of the Upstream Transmission Operon**

RPA analysis of the plasmid-borne *kilB* message was performed using the same riboprobe (Figure 2.3 A) as well as RNA isolated from *S. lividans* TK23 cells harboring the conjugative thiostrepton-resistant pIJ101 derivative pIJ303, cultures of which had been grown to increasing cell densities. Unexpectedly, a protected fragment of approximately 386 nt (again designated RT for readthrough transcription) appeared in a temporally increasing manner for TK23(pIJ303) (Figure 2.4, lanes 1-4) but was absent from a RPA involving strain NWR2 RNA (lane 5) as well as those using TK23(pIJ303) RNA pre-treated with RNase by the method described earlier (data not shown). An RNA fragment of this size for plasmid-containing cells indicated the existence of *kilB*-containing transcripts that must have initiated on pIJ303 within the upstream transmission operon at or before the position corresponding to the 3' end of the riboprobe, which as shown in Figure 2.3 A is the *SalI* site within *orf66*.



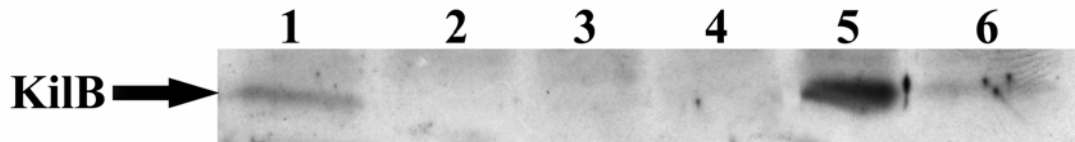
**Figure 2.4. Evidence for readthrough transcription across the *kilB* promoter region on pIJ101.** Total RNA was isolated from *S. lividans* strain TK23 harboring pIJ303 at increasing culture densities during growth in liquid YEME medium and then analyzed by RPA as described in the legend to Figure 3. Reactions in lanes 1-4 involved RNA isolated from cells harvested at an absorbance (600 nm) of 0.434, 0.819, 1.346 and 1.347 (12 h later than the culture at 1.346), respectively, while lane 5 involves RNA isolated from strain NWR2 grown to an absorbance (600 nm) of 1.393. A protected fragment of approximately 386 nt (which corresponds to readthrough transcription [RT] as indicated in Figure 3 A) is shown, and only this portion of the gel is included in the figure. The RPA experiment as presented was performed a total of three times and in each case more protected fragment corresponding to readthrough transcription was evident in late exponential and stationary phase growth than at earlier times assayed in exponential phase.

To what extent transcription initiating from the *kilB* promoter region also contributes to plasmid-borne *kilB* expression could not be ascertained from these RPAs performed with TK23(pIJ303) RNA because numerous additional fragments present throughout the gels below the RT fragment consistently obscured interpretation of results for potential P1-initiated and P2-initiated *kilB* messages (data not shown). Since only full-length riboprobe was used in RPA experiments (see Materials and Methods) and only reactions using TK23(pIJ303) RNA yielded these additional fragments, they appeared to have been formed during the RPA assay by occasional RNase cleavage at unknown

discrete locations within the RT fragment, probably when it was complexed to hybridizing cellular RNA. We nevertheless subsequently investigated the contribution of the P1 and P2 promoters to plasmid-borne *kilB* gene expression by primer extension analysis as detailed in a later results section.

One interpretation from the above RPA results is that the previously identified (Pettis and Cohen 1996) pIJ101 transcript that initiates upstream of the *tra* gene (Figure 2.1) and includes *tra*, *spdA*, *spdB*, and *orf66* actually extends to include the additional *kilB* gene. The correctness of this notion was confirmed by assessing insertion and deletion mutations within the upstream transmission operon for polar effects on downstream *kilB* gene expression. pIJ101 derivatives containing such mutations were introduced into *S. lividans* strain TK23, and broth cultures of the resulting strains were grown to equivalent cell densities, harvested, and then examined for production of KilB protein by Western blotting. Deletion of a small region upstream of *tra* including its promoter (Figure 2.1) on plasmid pIJ303, thereby creating plasmid pIJ303- $\Delta$ BglII-NruI, effectively eliminated KilB protein expression (compare Figure 2.5, lanes 1 and 2, which involve cell extracts containing pIJ303 versus pIJ303- $\Delta$ BglII-NruI, respectively); also, previously described (Kendall and Cohen 1987) pUC19 insertions (i.e., J20 and K22 in Figure 2.1) into either the *tra* or *spdA* genes on pIJ303 had similar effects on KilB expression (Figure 2.5, lanes 3 [pIJ303-J20] and 4 [pIJ303-K22]). Finally, insertion of a kanamycin-resistance cassette into *orf66* (i.e., N66 in Figure 2.1) on the conjugative hygromycin-resistant pIJ101 derivative pHYG3 resulted in a significant reduction in the intracellular concentration of KilB as compared to the wild type (compare Figure 2.5, lanes 5 [pHYG3] and 6 [pHYG3-N66]). This reduction in KilB concentration for

pHYG3-N66 was also accompanied by an approximate two-fold reduction in plasmid spread relative to the parental plasmid when transmission was assessed by a previously described (Pettis and Cohen 2000) replica plate assay (data not shown).



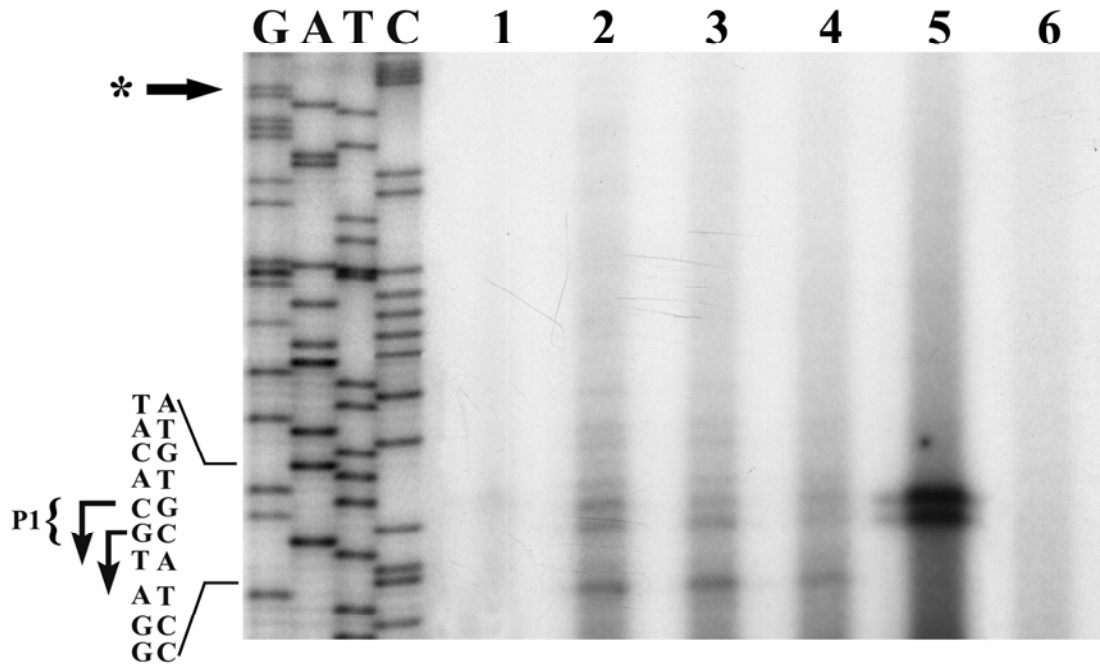
**Figure 2.5. Effects of transmission operon mutations on KilB protein expression.** Cultures of strain TK23 containing various insertion and deletion derivatives of the conjugative pIJ101 derivatives pIJ303 and pHYG3 were grown in YEME medium to approximately equivalent cell densities and then analyzed for KilB protein by Western blotting as described in the legend to Figure 3. Lanes: 1, pIJ303; 2, pIJ303- $\Delta BgIII-NruI$ ; 3, pIJ303-J20; 4, pIJ303-K22; 5, pHYG3; 6, pHYG3-N66.

In control Western blots (data not shown) using previously described antibodies raised against the pIJ101 KorA protein (Tai and Cohen 1994), none of the insertion mutations affected the normal intracellular level of KorA (the deletion derivative pIJ303- $\Delta BgIII-NruI$ , as expected, did not produce KorA since its deletion extends into the 5' end of *korA* as shown in Figure 2.1). These results along with other evidence (e.g., comparison of yields following plasmid isolation) suggest that none of the mutations have significant effects on either plasmid copy number or plasmid stability.

### **Evidence for Strong Repression of the Intercistronic *kilB* Promoter on pIJ101 by KorB**

While polarity exhibited by mutations within an operon is not a new phenomenon, the results here for pIJ101 were intriguing since transmission operon mutations were polar on the distal *kilB* gene despite the fact that the *kilB* promoter region remained intact. These results implied that expression from the intercistronic *kilB* promoter on pIJ101 may be strongly repressed by KorB under the non-mating conditions used here. To begin to examine this notion, transcription originating from this promoter region on pIJ101 was analyzed by primer extension analysis using the radiolabelled primer *kilBext* (see Materials and Methods for its sequence), which would yield extension products of 61 nt for the previously described start site for P1 and 106 nt for P2 (Stein et al. 1989). In line with the initiation sites seen using RPA for the *kilB* P1 promoter in strain NWR2 (Figure 2.3 C), weak reverse-transcribed products of 61 and 62 nt were observed for RNA extracted from TK23(pIJ303) cells grown to increasing cell densities (Figure 2.6, lanes 1-4), and these same extension products were also readily observable for strain NWR2 (lane 5) but not for TK23 alone (lane 6). While the temporal expression pattern for the *kilB* P1 promoter on pIJ303 here was similar to that when it was chromosomally inserted in strain NWR2 (Figure 2.3 C), the amount of transcription originating from the plasmid-borne promoter was significantly reduced. A comparison by densitometric scanning of lanes 2 and 5 of Figure 2.6, for example, which involved equivalent amounts of RNA isolated from TK23[pIJ303] and NWR2 cultures grown to similar cell densities, revealed a five-fold lower concentration of P1-initiated transcripts for plasmid-containing cells than for strain NWR2 (data not shown). Taking into account a copy number increase of at least 100-fold for *kilB* in cells harboring pIJ101 (Kieser et al. 1982), the observed

difference actually represents at least a 500-fold reduction in transcription initiation at the *kilB* P1 promoter on pIJ101 as compared to its unregulated counterpart in strain NWR2.



**Figure 2.6. Comparison by primer extension of P1-initiated *kilB* mRNA between pIJ101-containing *S. lividans* cells and strain NWR2.** Following annealing of radiolabeled primer *kilB*-xtn (see Materials and Methods for sequence) to 10  $\mu$ g of total RNA isolated from strain TK23 harboring pIJ303 at the timepoints (1-4) indicated in the legend for Figure 4, primer extensions were carried out as described previously (McDowall et al. 1994). Extension products were resolved on an 8% LongRanger sequencing gel in order in lanes 1-4 alongside a DNA sequence ladder of this pIJ101 region, which was generated using pGSP290 template and primer *kilB*-xtn; bands were then visualized by autoradiography. Lane 5 represents an identical reaction involving RNA isolated from a culture of strain NWR2 cells, which had been grown to an absorbance at 600 nm of 0.932, while lane 6 involves RNA isolated from TK23 grown to an absorbance of 1.357. The experiment as shown was performed a total of three times with identical results in each case. The asterisk denotes the expected position of fragments that would be indicative of P2-initiated transcription.

Here using primer extension, as with the RPA analysis for NWR2 earlier, there was no indication of transcription originating from the putative P2 promoter (whose start site is marked by an asterisk in Figure 2.6) for either TK23(pIJ303) (lanes 1-4) or again for NWR2 (lane 5) even upon extended autoradiography (data not shown). A few fainter bands of sizes larger than the P1-derived products but never equal in size to the expected P2-derived products were evident in TK23(pIJ303) reactions but not in those involving NWR2. These extended products thus do not appear to be derived from transcripts originating from within the *kilB* promoter region (since this is also present in its entirety in strain NWR2) and instead perhaps represent truncated extension products which were derived from premature termination of reverse transcriptase on operon transcript templates, the latter of which would be present exclusively in RNA derived from TK23(pIJ303) cells. Premature termination on operon transcripts may also account for the bands of sizes smaller than P1-derived products that appear for TK23(pIJ303) (lanes 1-4), but are not apparent for NWR2 (lane 5).

## **Discussion**

The *kilB* gene of *Streptomyces lividans* plasmid pIJ101 is essential for plasmid spreading. Because of the extremely adverse growth effects associated with unregulated KilB protein expression, its specific function may be either to inhibit recipient cell growth in order to prolong the temporally regulated process of pIJ101 transmission or it may instead contribute intrinsically to the uncharacterized plasmid spread mechanism and as a result indirectly cause some cell growth inhibition (Pettis et al. 2001). Here, we have demonstrated that all transfer and spread loci of pIJ101 including the *kilB* gene almost certainly constitute a single transmission operon that is coordinately expressed from the

*tra* promoter, which is under control of the pIJ101 KorA repressor protein. The distally located *kilB* gene is also under additional separate transcriptional regulation by the pIJ101 KorB protein, which binds and regulates expression at the intercistronic *kilB* promoter region. During mating, regulation by Kor proteins has been postulated to be interrupted by dilution of these repressors or by their temporary inactivation (Kendall and Cohen 1987, Hagege et al. 1999); interruption of Kor protein repression would then lead to pIJ101 transfer and spread gene induction and ultimately to the high efficiency transfer, spreading, and transient recipient cell growth inhibition that is typical for pIJ101 transmission. By being under the direct transcriptional control of two different repressors, putative induction of *kilB* gene expression during mating (as well as its subsequent repression once mating is completed) would be uniquely responsive to whatever factors modulate the function of either KorA or KorB or both. Induction of *kilB* expression would also presumably benefit from the presence of both the *tra* operon promoter as well the *kilB* promoter, the combination of which could result in an induced level of *kilB* expression that may be greater than that seen for the other transmission loci.

Since significant repression by KorB of the intercistronic *kilB* promoter was apparent under the non-mating conditions examined here, and optimal *kilB* gene expression on pIJ101 was shown to rely on transcription initiating upstream, most likely from the *tra* operon promoter, potential interactions between elongating RNA polymerase molecules synthesizing transmission operon transcripts and KorB repressor bound at the *kilB* P1 promoter are likely to be a key to understanding how *kilB* expression on plasmid pIJ101 is ultimately controlled. Our RPA analysis demonstrating readthrough transcription across the *kilB* promoter region shows that at least some elongating



polymerase must apparently displace KorB and continue synthesis into *kilB* sequences. Such displacement of bound KorB might also provide access for additional RNA polymerase to begin transcription at *kilB* P1. However, since site-specific DNA binding proteins including repressors have been shown to cause the efficient arrest and termination of ongoing mRNA synthesis in other bacteria (Uptain et al. 1997), there remains the possibility that some operon transcription on pIJ101 is similarly terminated when the streptomycete RNA polymerase encounters KorB protein bound at the *kilB* P1 promoter. In this case, KorB repressor would effectively function not only to prevent transcription initiation at *kilB* P1 but also to attenuate ongoing operon transcription (Uptain et al. 1997, Deuschle et al. 1986).

Previously we showed that KilB protein is present in relevant non-mating *S. lividans* cultures (Pettis et al. 2001), and this observation was extended here to show this production in pIJ101-containing cells is due to both limited transcription from the *kilB* P1 promoter as well as expression of *kilB* as part of the full transmission operon. As spread proteins are thought to participate in subsequent intramycelial transfer in recipient mycelia (Hopwood and Kieser 1993, Pettis et al. 2001), the significance *per se* of spread gene expression and protein production in non-mating streptomycete cells remains undetermined. It is possible that in addition to their presumed intramycelial role in the recipient, spread proteins may also somehow participate in Tra-mediated intermycelial transfer of pIJ101 plasmids from donor to recipient hyphae (Hopwood and Kieser 1993).

The concentration of KilB protein in *S. lividans* cells containing pIJ101 or in strain NWR2 was known to increase in a temporal manner throughout the life cycle and reach maximum amounts following sporulation during surface growth or during

stationary growth in liquid culture (Pettis et al. 2001). Although a posttranscriptional mechanism was shown here to be responsible for the temporal accumulation of KilB protein in strain NWR2, operon transcription reading into *kilB* on pIJ101 also appeared to show a temporal increase. Taken together, these results suggest that a combination of transcriptional and posttranscriptional mechanisms may ultimately contribute to the production and accumulation of plasmid-encoded KilB protein.

The RPA and primer extension analyses involving strain NWR2 here as well as our additional primer extensions involving cells harboring pIJ101 failed to reveal any *kilB* transcription initiating from the weaker unregulated promoter previously described by Stein et al. (1989), which we have designated P2. In their study, this putative promoter was identified using primer extension of RNA isolated from plasmid-containing cells in which the *kilB* promoter region was cloned onto a promoter probe vector. Although the exact reason for the discrepancy between the previous study and ours remains undetermined, it may be noteworthy that the primer extension endpoint determined previously (i.e., P2 initiation site in Figure 2.1) is positioned near the 3' end of the inverted repeat that closely follows *orf66*. As this sequence may be capable of forming a hairpin in mRNA, a possible explanation for the previous observation is that P2 represented the position where extending reverse transcriptase molecules were halted due to the formation of a potential secondary structure on transcripts that had actually initiated at a relatively low frequency within upstream vector sequences; in line with this argument, Stein et al. reported some apparent readthrough transcription for their promoter probe vector in the absence of cloned promoter sequences (Stein et al. 1989).

Fortuitously, no such readthrough transcription from upstream chromosomal sequences was apparent here for our studies involving strain NWR2.

The ability of the pIJ101 KorB repressor to regulate transcription from the *kilB* promoter has been well documented (Hopwood and Kieser 1993). Also known previously was the intriguing capacity of KorA to override or prevent lethal effects of the singly cloned unregulated *kilB* gene (Kendall and Cohen 1987); however, since the KorA repressor does not directly control expression from the *kilB* promoter (Stein et al. 1989), this particular regulatory capability of KorA has remained undetermined but has been postulated to represent a type of poison-antidote interaction involving the toxic KilB protein and KorA (Hopwood and Kieser 1993). In this study, we have demonstrated the existence of a post-transcriptional component for regulating the amount of KilB protein in cells and have presented data also strongly supporting the inclusion of the *kilB* gene as part of the pIJ101 transmission operon, which is under direct transcriptional control by KorA. This multitude of diverse regulatory mechanisms now known to affect KilB expression, accumulation and possibly function underscores the apparent necessity for careful monitoring of this potentially lethal plasmid spread component.

## **Materials and Methods**

### **Bacterial Strains and Plasmid Constructions**

*S. lividans* strain TK23 was described previously (Kieser et al. 2000) as was strain NWR2 (Pettis et al. 2001). The *E. coli* host for propagation of plasmid constructs was DH10B [ $F^-$  *mcrA*  $\Delta$ (*mrr-hsdRMS-mcrBC*)  $\phi$ 80*dlacZ* $\Delta$ M15  $\Delta$ *lacX74 deoR recA1 endA1 araD139*  $\Delta$ (*ara-leu*)7697 *galU galK* $\lambda^-$  *rpsL nupG*] (Life Technologies Inc.). Plasmids pIJ303-J20 and pIJ303-K22 have been described elsewhere (Kendall and Cohen 1987),

while pIJ303- $\Delta$ *Bgl*III-*Nru*I was constructed by first digesting pIJ303 with *Bgl*III and *Nru*I, then making the *Bgl*III ends flush with Klenow fragment and nucleotides, and finally ligating the resulting blunt ends together. Plasmid pHYG3-N66 was constructed by first inserting the 4.0 kb *Bam*HI-*Pvu*II fragment of pHYG3 (Pettis and Cohen 1994) into these sites on pSP72 (Promega). This plasmid (pGSP285) was linearized by partial digestion with *Nae*I and ligated to a 1.7 kb *Nhe*I-*Bam*HI fragment conferring kanamycin resistance (derived from plasmid pCAO106) (Omer et al. 1988), which was made blunt ended by the method described above prior to ligation. The resulting clone (pGSP286) containing this insert at the *Nae*I site in *orf66* was isolated, and the 5.7 kb *Bam*HI-*Pvu*II fragment from pGSP286 was then ligated to the 6.5-kb *Bam*HI-*Pvu*II fragment of pHYG3 in order to create pHYG3-N66. pSCON33 contains the 390-bp *Ava*I-*Sal*I region of pIJ101 containing the *kilB* gene cloned as a *Ava*I (blunt-ended)-*Sal*I fragment into the *Eco*RV-*Sal*I sites of pSP72. Plasmids pSCON2 (Ducote and Pettis 2003) and pGSP290 (Pettis and Prakash 1999) have been described previously.

### **Bacteriological and Molecular Biological Techniques**

Transformation of *E. coli* (Sambrook et al. 1989) and *S. lividans* (Kieser et al. 2000) were performed as described. Ampicillin was used at 50  $\mu$ g/ml for selection of plasmids in *E. coli*, while thiostrepton (50  $\mu$ g/ml), hygromycin (200  $\mu$ g/ml), spectinomycin (400  $\mu$ g/ml), and streptomycin (30  $\mu$ g/ml) were used where appropriate at the indicated concentrations to select for plasmids in *S. lividans* on agar media, or these same antibiotics were used at one-tenth the indicated concentrations in liquid (Kieser et al. 2000). To obtain sufficient quantities of spores, *S. lividans* transformant colonies were excised, processed and re-plated as described previously (Pettis et al. 2001).

Replica plate assays for determining plasmid transmission were carried out as described (Pettis and Cohen 2000). Liquid cultures of *S. lividans* were grown in yeast extract-malt extract (YEME) medium (Kieser et al. 2000).

Cloning was performed using customary procedures described elsewhere (Sambrook 1989), and DNA sequences were determined by manual sequencing using a Sequenase version 2.0 sequencing kit (USB Corporation) with relevant primers. Autoradiography of radioactive gels and blots was done at -70°C using Hyperfilm MP (Amersham Pharmacia Biotech) with intensifying screens. Western blotting of KilB was performed as previously described (Pettis et al. 2001). (Pettis et al., 2001)

For catechol dioxygenase (XylE) assays, relevant *S. lividans* liquid cultures were grown to an absorbance at 600 nm of approximately 0.1, where they were diluted several fold into fresh medium (this was designated time 0). At the subsequent times indicated in the text, 1.5 ml of mycelia were harvested by centrifugation, washed in 20 mM potassium phosphate buffer, pH 7.2, and the pellets were frozen at -20° C. Assays were performed and catechol dioxygenase activity was quantified as described (Ingram et al. 1989) using the rate of change in absorbance at 375 nm obtained over a six min time range.

### **RNA Isolation and Analysis**

RNA was isolated using TriReagent (Molecular Research Center, Inc.) along with slightly modified manufacturer's instructions. See Appendix B. Briefly, mycelia were harvested from liquid cultures in 1.5 ml tubes and vortexed vigorously in 500 µl TriReagent containing approximately 300 mg of 0.1 mm Zirconia/Silica beads (Biospec Products, Inc.) for 5 min; then an additional 500 µl of TriReagent was added, and samples were incubated for a further 5 min at room temperature. Cell debris was pelleted

in a microfuge at 14,000 rpm for 2 min, supernatants were then transferred to a fresh tube, and the remainder of the RNA purification procedure was performed according to the manufacturer. To eliminate the possibility of DNA contamination, RNA was treated with DNase I, extracted with phenol:chloroform (50:50) and then chloroform, and it was finally ethanol precipitated with subsequent resuspension in DEPC-treated water. The absorbance at 260 nm of each sample was determined to calculate RNA concentrations, and RNA integrity was determined by agarose gel electrophoresis as described (Sambrook et al. 1989).

For dot blot hybridizations, 5 µg of total RNA was bound to Hybond nylon membrane (Amersham Pharmacia Biotech) by vacuum blotting and then crosslinked to the blot using ultraviolet light as described (Sambrook et al. 1989). A *xyIE*-specific radiolabeled probe was created using the NEBlot random priming kit (New England Biolabs) along with [ $\alpha$ -<sup>32</sup>P]dATP (600 Ci/mmol) and PCR-amplified template consisting of the entire *xyIE* ORF. Hybridizations using approximately 1 x 10<sup>6</sup> cpm of probe were performed as described elsewhere (Brasch et al. 1993).

Riboprobes of 407 nt for the RPA were generated by *in vitro* transcription using a Maxiscript T7 kit (Ambion), along with linearized plasmid pSCON33 digested at *SalI* as a template and [ $\alpha$ -<sup>32</sup>P]CTP (800 Ci/mmol). Unincorporated ribonucleotides were removed using a Microspin S-400 HR column (Amersham Pharmacia Biotech), and full-length probe was isolated on a 7% denaturing polyacrylamide gel, excised with a scalpel and eluted overnight into the elution buffer provided in a RPAIII kit (Ambion). RPAs were performed using the reagents provided in this kit and as described (Pettis and Cohen 1996), except that following the hybridization step reactions were treated with RNaseA

only, and the final products were resolved on an 8% denaturing Long Ranger (BioWhittaker Molecular Applications) sequencing gel alongside an unrelated DNA sequencing ladder.

For primer extension analysis, primer *kilB*-xtn, 5'-CGATCACGGCAATGAGCG TGGTCAC-3', was end-labeled using T4 polynucleotide kinase and [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci/mmol) as described (Sambrook et al. 1989). Primer extensions were performed as described previously (McDowall et al. 1994) except that 10 µg of total RNA was used per reaction, and completed reactions were resolved on an 8% Long Ranger sequencing gel alongside the sequence of pSCON33, which was generated by manual sequencing as described above using primer *kilB*-xtn. Following autoradiography, densitometric analysis of bands present on exposed film was performed using Quantity One analysis software from Bio-Rad.

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**CHAPTER THREE:**

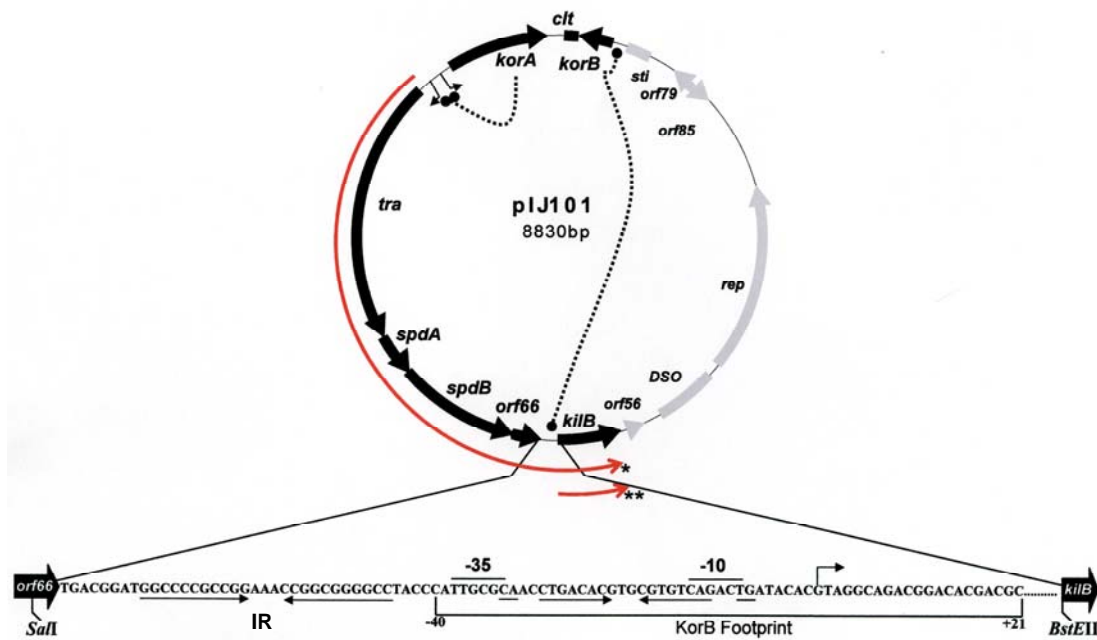
**EXPRESSION OF THE pIJ101 *kilB* GENE: TERMINATION AND  
ANTITERMINATION MECHANISMS REGULATE A POTENTIALLY LETHAL  
PLASMID FUNCTION**

## Introduction

The Gram-positive bacterial genus *Streptomyces* distinguishes itself from other prokaryotes with numerous extraordinary characteristics that have fascinated investigators since 1949. With a complex life style unique among bacteria, an extremely efficient and apparently novel conjugal mechanism and the production of thousands of chemicals vital to all aspects of industry and medicine, *Streptomyces* biology has left an indelible mark on the world. Perhaps as interesting as the above features are the mechanisms that govern them. Gene regulation has always been known to play an important role in *Streptomyces* development but not until the sequence of the model streptomycete *S. coelicolor* A3(2) was the scope of the *Streptomyces* regulome realized. With an extraordinary number of genes with putative regulatory function including 65 RNA polymerase sigma factors, three times the previous record holder, two-component regulators and hundreds of secreted proteins (Bentley et al. 2002), *Streptomyces* places an unprecedented emphasis on regulation.

The emphasis on regulation is reflected in the transfer and spread region of the streptomycete plasmid pIJ101 (Figure 3.1). One of the most widely characterized plasmids of streptomycetes, pIJ101 is an 8.8 kb, autonomously replicating circular plasmid that is capable of transferring itself to up to 100% of all potential recipients as well as promoting the transfer of chromosomal markers while remaining an autonomous entity. An interesting feature of streptomycete plasmids is the relatively few plasmid-encoded functions that participate in plasmid transmission. pIJ101 is no exception possessing only two plasmid loci, *tra* (Kendall and Cohen 1987) and *clt* (Pettis and Cohen 1994), essential to plasmid transfer with three additional functions, *spdA*, *spdB*

and *kilB* (Kendall and Cohen 1987) contributing to plasmid transmission through a poorly defined process termed spread.



**Figure 3.1. Genetic organization and physical map of pIJ101 including the entire transmission operon transcript and separate *kilB* transcript.** Genetic loci (boxes) and open reading frames (arrows) are shown with the direction of translation indicated. The replication and stability region (grey arrows and boxes) is separated from the transfer and spread region (back arrows and boxes). The Kor functions are indicated by dotted lines leading to their target and the entire transfer and spread operon (\*), including *kilB*, is indicated along with the detached *kilB* transcript (\*\*). The enlarged region spans the intergenic space between *orf66* and *kilB* (Kendall and Cohen 1988) and includes the *kilB* promoter elements (Stein et al. 1989), an inverted repeat (IR) region, the KorB footprint (Ducote and Pettis 2003) and relevant restriction sites.

The *tra*, *spdA* and *spdB* genes, the open reading frame *orf66* (Kendall and Cohen 1988), and the *kilB* (Schully and Pettis 2003) gene comprise the transmission operon (Figure 3.1), which produces all of the plasmid-encoded proteins involved in plasmid transfer and spread of pIJ101 (Kendall and Cohen 1987). The *tra*, *spdA*, *spdB* and *orf66* open reading frames are adjoined on the operon message, which is present in varying quantities throughout *Streptomyces* growth (Pettis and Cohen 1996, Schully and Pettis 2003). *kilB* can be expressed as part of the transmission operon (Figure 3.1, indicated with a single asterisk\*) (Schully and Pettis 2003), whereon it is separated from the other operon genes by a 105-bp intercistronic region (Kendall and Cohen 1988). Readthrough transcription through the intercistronic region appears to steadily increase throughout development (Schully and Pettis 2003). *kilB* can also be expressed as an unattached transcript originating from its own promoter (Figure 3.1, indicated by two asterisks\*\*) (Buttner and Brown 1987, Stein et al. 1989), and expression from the *kilB* promoter increases during exponential phase and declines through stationary phase (Schully and Pettis 2003). Regulating the transcription of the transfer and spread functions are KorA, which negatively regulates transcription of the transmission operon from the *tra* promoter and KorB (Stein et al. 1989, Stein and Cohen 1990) which binds to the *kilB* promoter region (Figure 3.1) and protects an unexpectedly large 60-base-pair stretch (Ducote and Pettis 2003) and in so doing represses the distal operon gene *kilB* (Figure 3.1) by approximately 500-fold (Schully and Pettis 2003).

Earlier Western blot analyses demonstrated that the Tra and KilB proteins have contrasting expression patterns. Pettis and Cohen (1996) observed Tra at its highest concentration at the earliest timepoint analyzed followed by a rapid reduction until it was

no longer detectable by 24 hrs, a time which was coincident with aerial hyphae formation and the completion of mating. Steady state concentrations of KilB protein progressively increased throughout cellular development whether the *kilB* gene was contained on a pIJ101 derivative or was integrated into the *Streptomyces lividans* chromosome (Pettis et al. 2001), and KilB production or accumulation was found to be governed at least in part by a post-transcriptional control mechanism (Pettis and Schully 2001, Schully and Pettis 2003).

In the previous chapter, we sought to define the mechanisms that govern the temporally increasing expression profile of the KilB protein. We found that the transmission operon of the streptomycete plasmid pIJ101 exemplifies an unusual operon wherein regulation of the distal gene (*kilB*) is tightly controlled by multiple modes of transcriptional regulation imposed throughout the entire operon. In addition to temporally regulated activity (Figure 2.2), the internal promoter is substantially repressed by the negative transcriptional regulator KorB (Figure 2.6). Transcription of the *kilB* gene is in fact dependent upon operon transcription reading through the intergenic region between *orf66* and *kilB* as mutations introduced into upstream sequence have polar effects on KilB expression (Figure 2.5). Readthrough transcription then expresses *kilB* as part of the transmission operon (Figure 2.4) as well as potentially displacing KorB to allow initiation to occur from the *kilB* promoter (Figure 2.6).

Here, we further define the events that transpire within the intergenic region as the transcribing RNA polymerase encounters KorB bound to the *kilB* operator. Using S1 mapping, we found that in addition to readthrough transcription, termination of operon transcription occurs in the intergenic region. The site of termination consistently

occurred in the region preceding the 5' end of the KorB footprint (Figure 3.1) (Ducote and Pettis 2003), implying that KorB was involved in the transcription termination event. This notion was determined to be accurate using inducible constructs that mimic expression of the transmission operon in the presence and absence of KorB suggesting that KorB may act as a physical barrier to operon transcription. Interestingly, transcription readthrough and termination appear to be under the control of a previously undefined regulator as well. Mutations in and around a 25-nucleotide inverted repeat (Figure 3.1, IR) located immediately after *orf66* abolished operon readthrough and KilB protein expression indicating that antitermination is working in opposition to the roadblock imposed by KorB.

## **Results**

### **Operon Transcription Terminates in the Intergenic Region Between *orf66* and *kilB***

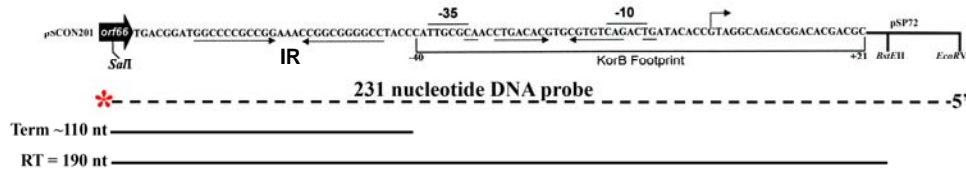
As described in chapter two, the demonstration of transcripts originating from the pIJ101 plasmid-borne *kilB* promoter indicates that, although severely repressed by KorB, the *kilB* promoter is active. However, the dramatic polar effect that mutations introduced into upstream sequences have on KilB protein suggests otherwise. When considered together with the demonstration of readthrough transcription, these data suggest a mechanism by which the *kilB* promoter is largely repressed by the KorB protein and transcription from the upstream operon reads through the intergenic region expressing *kilB* as part of the transmission operon. This model suggests a physical interaction between KorB and the transcribing RNA polymerase, an interaction that at some frequency may displace KorB, allowing readthrough and perhaps transcription initiation at the *kilB* promoter. One can also imagine termination occurring as a result of this



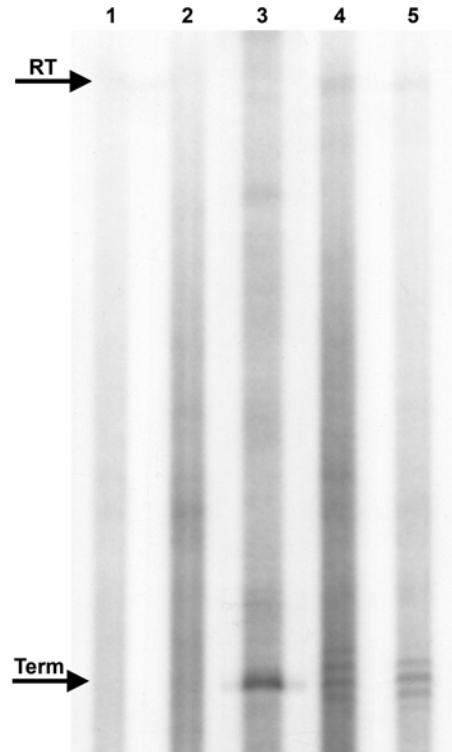
interaction as well (Schully and Pettis 2003). To examine the possibility that KorB is capable of terminating transcription in the intergenic region of the transmission operon, we sought first to define the hypothetical 3' end of this truncated operon transcript using S1 mapping. Here, a 231-nucleotide 3'-end-labeled DNA probe (Figure 3.2 A) complementary to the readthrough transcript was hybridized with RNA isolated from cultures of *Streptomyces lividans* strain TK23 harboring pHYG3, which had been harvested at various times during growth in liquid media. To differentiate between full length transcripts and truncated transcripts, the probe extended from the *BstEII* site at the beginning of the *kilB* ORF to the *SalI* site within *orf66* (Figure 3.1) (Kendall and Cohen 1988). If termination occurs as a result of KorB, it would likely take place in the vicinity of the 5' end of the KorB footprint (i.e., -40 relative to the *kilB* promoter, Figure 3.1) (Ducote and Pettis 2003); thus, a protected fragment of approximately 110 nucleotides would represent a truncated transcript, while protected fragments of 190 nucleotides would represent readthrough transcription. Because the probe was labeled at the 3' end, transcripts originating from the *kilB* promoter would not be detected.

S1 mapping performed on RNA derived from cultures of *S. lividans* strain TK23 harboring pHYG3 (Figure 3.2 B, lanes 3-5) yielded multiple protected fragments of the sizes expected to indicate truncated operon transcripts (Figure 3.2 B, designated Term for termination). The site(s) of termination are conspicuously located in the right arm of an inverted repeat (IR) (Kendall and Cohen 1988) predicted to produce a formidable stem-and-loop structure in the readthrough transcript and just prior to the *kilB*-promoter-proximal end of the KorB footprint (Figure 3.2 A) (Ducote and Pettis 2003). The pattern

A.



B.



**Figure 3.2. Attenuation of the transmission operon transcript.**

A) 3'-end-labeled DNA probe for the detection of truncated operon transcripts. The 231-base-pair *SalI-EcoRV* fragment from pSCON201 was filled in with Klenow fragment in the presence of [ $\alpha$ - $^{32}$ P]-dATP and [ $\alpha$ - $^{32}$ P]-dCTP. Of the 231 nucleotides present in the probe, 190 nucleotides are complementary to the readthrough transcript; the protected readthrough transcript (RT) is shown beneath along with the presumed size of a truncated transcript (Term).

B) S1 nuclease mapping of intergenic region of operon transcript. 50  $\mu$ g of total RNA isolated from TK23 (lanes 1 and 2) or TK23 harboring pHYG3 (lanes 3-5) at increasing timepoints ( $OD_{600} = 0.8$  and  $1.394$  for lanes 1 and 2,  $OD_{600} = 0.664$ ,  $0.919$  and  $1.512$  for lanes 3-5) was hybridized to the probe depicted in (A) and subjected to S1 nuclease digestion as described (Kieser et al. 2000). The resulting fragments were resolved in the order presented on an 8% denaturing polyacrylamide gel next to a DNA sequencing ladder and exposed to film. Based on sizes determined by comparison to the DNA ladder, protected fragments of 190 nucleotides (RT) were detected as were fragments ranging from 101-105 nucleotides (Term).

indicates a discrete site for termination occurring during the earliest exponential timepoint taken (Figure 3.2 B, lane 3) and at least four sites for termination at later times during exponential (lane 4) and stationary (lane 5) phases. Additionally, a protected fragment of 190 nucleotides (Figure 3.2 B, designated RT for readthrough transcription), which indicates readthrough transcription, was also observed and displayed an increasing pattern similar to that observed previously (Schully and Pettis 2003). These protected fragments were absent in RNA isolated from TK23 alone (Figure 3.2 B, lanes 1 and 2) and from TK23(pHYG3) RNA that was digested with RNase (Data not shown). The observation of termination occurring in the intergenic region suggests that in addition to the numerous modes of regulation governing the expression of *kilB*, attenuation of the operon transcript prior to the *kilB* gene is also at work.

### **KorB Is an Attenuator of Operon Readthrough Transcription**

The observation that termination of the operon transcript is occurring at the *kilB*-promoter-proximal end of the KorB footprint (Ducote and Pettis 2003) implies that KorB is acting as a physical barrier to transcription elongation. Because of the lethal effects of unregulated *kilB* (Kendall and Cohen 1987), it was not possible to perform the previously described S1 mapping assay on a pIJ101 replicon lacking *korB*. It was therefore necessary to utilize artificial constructs in lieu of pIJ101 derivatives. To examine the termination potential within the intercistronic region in the presence and absence of KorB, we constructed pSCON93 (Figure 3.3 A), an artificial model for readthrough-dependent expression downstream of the KorB-repressed *kilB* promoter, and pSCON278, a *korB*<sup>-</sup> derivative of pSCON93.

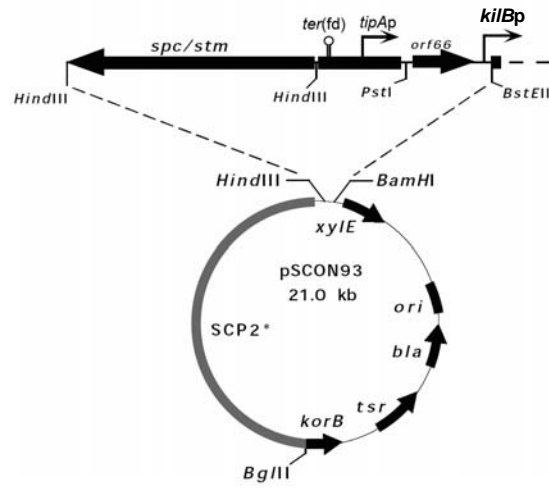
**Figure 3.3. Evidence for a KorB-mediated transcription roadblock.**

**A)** Genetic organization and physical map of pSCON93. pSCON93 contains the *korB* gene inserted at the *Bgl*III site of pSCON2 (Ducote and Pettis 2003, Schully and Pettis 2003), along with the thiostrepton-inducible promoter *tipAp*, the transcription terminator from bacteriophage fd and the spectinomycin/streptomycin resistance cassette all inserted upstream of the *kilB* promoter region as indicated.

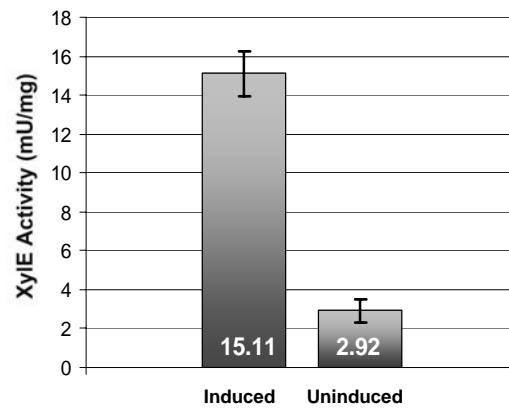
**B)** Average catechol dioxygenase (XylE) activity from *S. lividans* strain 1326 cultures harboring pSCON93 in the presence (induced) or absence (un-induced) of thiostrepton. Cultures were inoculated into YEME containing 40  $\mu\text{g ml}^{-1}$  spectinomycin and grown overnight. Upon evidence of visible growth, the cultures were split into two, diluted back to the original volume in media containing 40  $\mu\text{g ml}^{-1}$  spectinomycin. To one culture, inducer (thiostrepton) was added and to the other it was not. The cultures were then grown to mid-log phase and mycelia were harvested either to determine the absorbance of the culture at 600nm or to prepare extracts to measure XylE activity as previously described (Ingram et al. 1989, Schully and Pettis 2003). The average XylE activity is presented as mU/mg of total protein and the standard error is provided.

**C)** S1 mapping of readthrough transcription. RNA was isolated from mycelia of *S. lividans* strain 1326 (lane 1) or strain 1326 harboring pSCON93 (lane 2) or pSCON278 (lane 3) grown and induced as described above and was then subjected to S1 protection as described in the legend to Figure 3.2.

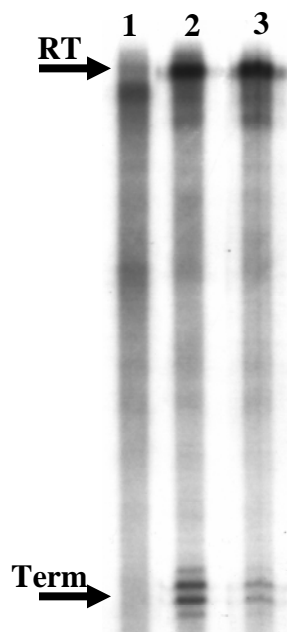
A.



B.



C.



pSCON93 (Figure 3.3 A) is a derivative of pSCON2 (Ducote and Pettis 2003, Schully and Pettis 2003) which contains the *korB* gene cloned at the *Bgl*III site. Upstream of the *kilB* promoter region lies *tipAp*, a promoter which is inducible by the antibiotic thiostrepton in *S. lividans* strain 1326 (Kieser et al. 2000) and which represents the *tra* promoter in this system. In order to select for the plasmid while the system remained in an uninduced state, the spectinomycin and streptomycin resistance cassette was added upstream and in the opposite orientation of *tipAp* and was separated from the *kilB* promoter region by the bacteriophage fd terminator [Figure 3.3 A, *ter*(fd)] to prevent vector transcription from reading into the region. pSCON278 is identical to pSCON93 except that it lacks the *korB* gene.

We validated pSCON93 as a suitable model for readthrough-dependent expression of a KorB-controlled gene. Plasmid pSCON93 was introduced into *S. lividans* strain 1326 (Kieser et al. 2000) by transformation and spores of transformants were inoculated into 25 ml of YEME containing 40  $\mu\text{g ml}^{-1}$  spectinomycin. The culture was grown to an absorbance at 600 nm of 0.1 at which time it was split into two and diluted back to the original volume with media containing spectinomycin (40  $\mu\text{g ml}^{-1}$ ), and 5  $\mu\text{g ml}^{-1}$  thiostrepton for the “induced” culture or only spectinomycin for the “uninduced” culture. These cultures were then grown to optical densities previously identified to demonstrate the highest levels of *kilB* promoter activity (i.e., absorbance at 600nm = 0.2 - 0.8 [Schully and Pettis 2003]) and then assayed for catechol dioxygenase (Xyle) activity as described (Schully and Pettis 2003, Ingram et al. 1989). Previously, Xyle activity produced from the unregulated *kilB* promoter during mid-exponential phase was determined to be 147 mU/mg (Schully and Pettis 2003). In the absence of upstream

transcription XylE activity from pSCON93 averaged only 2.92 mU/mg  $\pm$  0.79 (Figure 3.3 B). Interestingly, this value was actually two-fold lower than the XylE activity produced by the promoterless *xylE* vector pXE4 during exponential phase (Schully and Pettis 2003), which demonstrates quantitatively the considerable, if not absolute, level of repression that KorB exerts on the *kilB* promoter. Following the addition of inducer (thiostrepton), XylE activity increased 5-fold to 15.11 mU/mg  $\pm$  1.15 (Figure 3.3 B). 21 replicates of the assay were performed for induced samples and 18 for uninduced; the results were found to be reproducible and significant ( $p < 0.0001$ ).

To examine the potential for KorB-mediated termination of *tipAp*-initiated transcription, RNA was harvested from cultures of *S. lividans* strain 1326 alone or 1326 harboring pSCON93 or pSCON278, which had been induced as described above, and these RNAs were then subjected to the S1 protection assay as described in the previous section. In the presence of KorB (i.e., pSCON93), readthrough was observed as well as termination products (Figure 3.3 C, lane 2), with the latter occurring at the same positions and displaying a similar pattern to that observed for pHYG3 at later timepoints (Figure 3.2 B). In the absence of KorB (i.e., pSCON278) protected fragments indicative of readthrough were observed to be the predominant product and only following prolonged exposure did minor termination products begin to become apparent (Figure 3.3 C, lane 3). These fragments were not evident in RNA derived from *S. lividans* strain 1326 alone (Figure 3.3 C, lane 1). The demonstration of termination occurring predominately in the presence of KorB is consistent with the notion that in addition to repressing transcription from the *kilB* promoter, KorB is participating in the attenuation of transcription by acting as a physical barrier to the operon transcription elongation complex.

### **Antitermination Opposes KorB-mediated Termination**

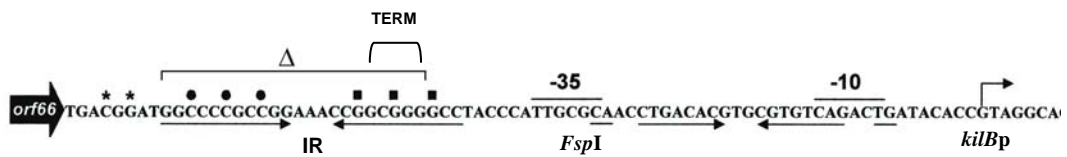
The position of operon transcript termination in the intercistronic *kilB* promoter region occurs not only immediately prior to the region of KorB binding (Ducote and Pettis 2003), but also overlapping an IR region (Figure 3.4 A). This latter region is predicted to form a perfect hairpin of eleven G-C base pairs (Figure 3.4 B) in the readthrough transcript with an energetically favorable free energy of formation (i.e.,  $\Delta G = -26.2$ ). To begin to investigate the potential role of this sequence in operon and/or *kilB* expression, we constructed a number of mutations in and around the inverted repeat. As there were no convenient restriction sites in the area, two nucleotides (Figure 3.4 A, designated by asterisks\*) were changed by a two-stage PCR-based site-directed mutagenesis method (described in Figure 3.5) in the intergenic region, which resulted in the creation of a *NsiI* site (ATGCAT) immediately downstream of the stop codon of *orf66*. *NsiI* was chosen because it is unique to pIJ101 and therefore could be used for additional manipulations in this area. When the resulting plasmid, pHYG3-*NsiI* (Figure 3.4 B) was analyzed for KilB protein expression by Western blotting as previously described (Pettis et al. 2001), we observed the level of KilB protein produced by this plasmid to be severely diminished (Figure 3.6 A, lane 3), suggesting that the sequence surrounding the inverted repeat may be important for antitermination, rather than termination of operon transcripts.

Consistent with the notion that the IR sequence forms a hairpin in the mRNA that somehow promotes antitermination of operon transcription, a number of additional mutants were constructed (Figure 3.4 B) including pHYG3-MUThp, which contains a mismatch every third base of the potential hairpin, an effect that raises the free energy of



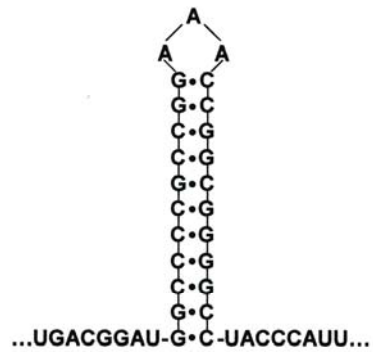
**Figure 3.4. Mutations introduced within and near the inverted repeat region located downstream of *orf66* and the effects of these mutations on putative hairpin formation in the transmission operon mRNA.** The sequence depicted above (A) spans the intergenic region between *orf66* and *kilB* and indicates the position of mutations introduced into the IR region. \*, mutations that produced the *NsiI* site; ●, mutations that produced pHYG3-MUThp; ■, mutations that produced pHYG3-MUThpX2; Δ, sequence deleted to produce pHYG3-Δhp. (B) The transcribed sequences and predicted structure of the wild type hairpin and each mutant are depicted with the calculated free energy of formation ( $\Delta G$ ) given below each structure. In (A), the positions of termination from wild type sequence are indicated with a bracket (Term, for termination).

A.



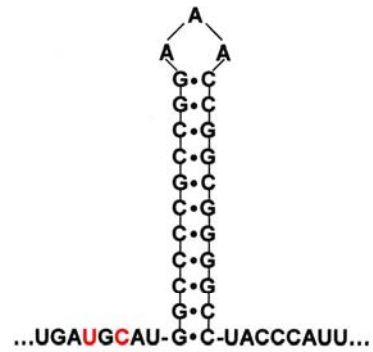
B.

**pHYG3**



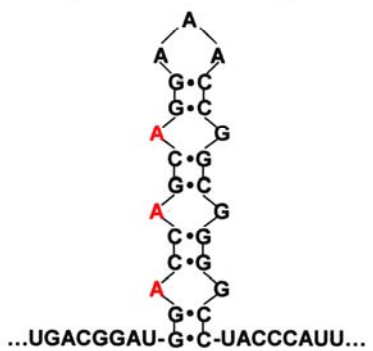
$\Delta G = -26.2$

**pHYG3-NsI**



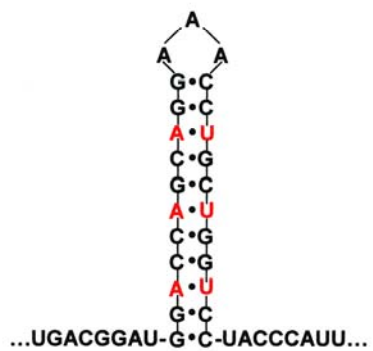
$\Delta G = -26.2$

**pHYG3-MUThp**



$\Delta G = -7.3$

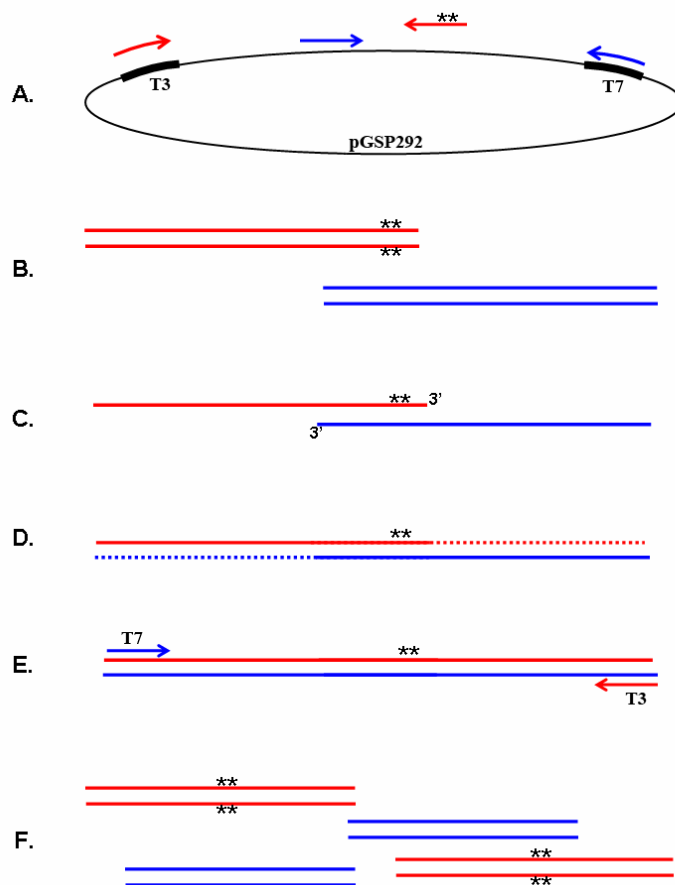
**pHYG3-MUThpX2**



$\Delta G = -22.9$

**pHYG3- $\Delta$ hp**



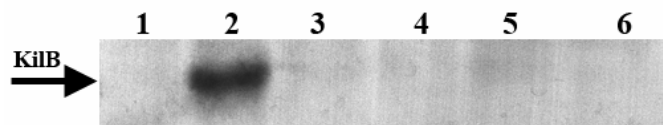


**Figure 3.5. PCR-based site-directed mutagenesis.** Here, two PCR reactions are performed, one employing a mutagenic primer containing the desired mutation (designated by asterisks in part A) producing two overlapping products (B), one of which contains the desired mutation. (C) The two products are combined, denatured and allowed to re-anneal producing a hybrid. (D) Additional nucleotides are added along with Klenow fragment which primes off of the 3' ends of the hybrid and produces double-stranded full length product. (E) The resulting double-stranded DNA is used as a template in a second PCR reaction using the outermost primers utilized in the first set of reactions. (F) The final reaction results in a pool of products that contain both wild type and mutant sequences, which are cloned and screened for the desired mutation.

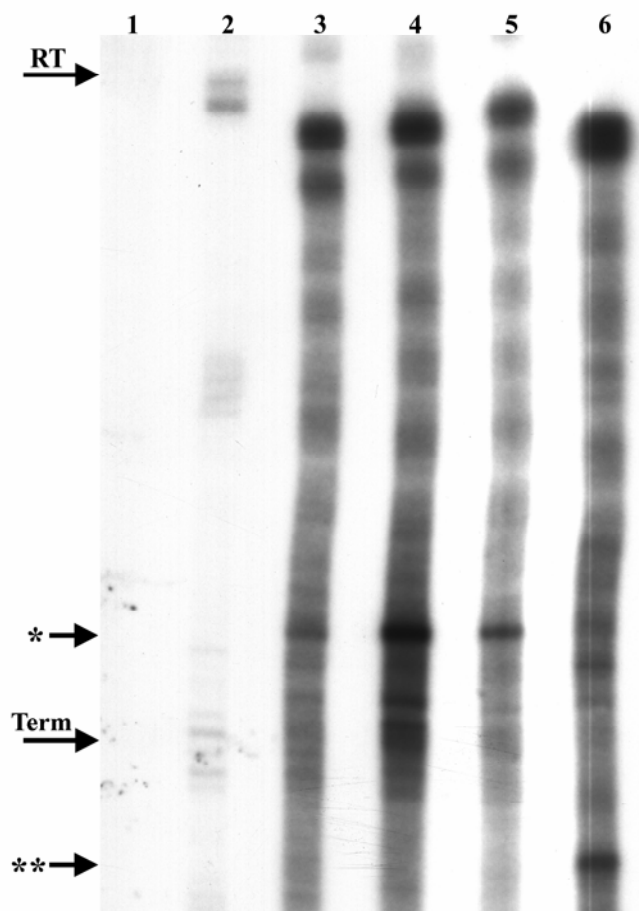
formation to  $\Delta G = -7.3$  and thereby reduces the likelihood the hairpin will form. pHYG3-MUThpX2 contains the compensating mutations to the mismatches found in pHYG3-MUThp, restoring the free energy of formation to  $\Delta G = -22.9$  and allowing us to assess the sequence specificity of the putative hairpin for antitermination. Finally, pHYG3- $\Delta$ hp is a deletion of 25 base pairs, which removes the symmetrical region entirely. Plasmids carrying the intergenic variants were introduced into *S. lividans* strain TK23 by transformation (Kieser et al. 2000), and transformants were grown in liquid culture to stages of development where KilB protein is known to be found in high concentrations (Pettis et al. 2001). At equivalent timepoints, cultures were harvested and assayed for KilB protein production by Western blotting and for readthrough transcription by S1 mapping as described earlier (except that the probes were generated from plasmids containing the appropriate mutations).

S1 mapping of RNA isolated from cultures harboring mutant plasmids produced significant background throughout the region of interest and so each mutation's affect on termination could not be clearly ascertained. The band corresponding to operon readthrough transcription (Figure 3.6 B, position indicated by RT), which was present in RNA isolated from cultures harboring pHYG3 (Figure 3.6 B, lane 2), was clearly absent from cultures harboring each of the mutant plasmids (Figure 3.6 B, lanes 3-6), indicating that the mutations eliminated readthrough transcription. Consistent with the earlier results demonstrating the necessity for readthrough transcription for KilB protein expression (Schully and Pettis 2003), all four mutations also reduced KilB protein expression to below the level of detection by Western blot (Figure 3.6 A).

A.



B.



**Figure 3.6. Evidence for antitermination of readthrough transcription.**

**A)** Effects of intergenic mutations on KilB protein expression. *S. lividans* strain TK23 (lane 1), TK23 harboring pHYG3 (lane 2), pHYG3-*Nsi*I (lane 3), pHYG3-MUThp (lane 4), pHYG3-MUThpX2 (lane 5) or pHYG3- $\Delta$ hp (lane 6) were grown to equivalent culture densities and harvested for Western blot analysis as previously described (Pettis et al. 2001, Schully and Pettis 2003).

**B)** S1 mapping of intergenic mutants. Additional mycelia were harvested from the cultures described above and RNA was isolated as described (Kieser et al. 2000). The resulting RNA was then subjected to S1 protection as described in the legend for Figure 3.2 and the products electrophoresed in the order described in (A).

## Discussion

In the previous chapter, we demonstrated that the transfer and spread functions of pIJ101 comprise a unique operon in which multiple modes of transcriptional and posttranscriptional control are coordinated to regulate the distal gene (*kilB*), a potentially lethal function that is essential for widespread dissemination of pIJ101. Our data suggest a model for operon transcription that involves transcription readthrough and suggests a physical interaction between the readthrough transcription complex and KorB bound to the *kilB* promoter. What happens to the RNA polymerase transcription elongation complex in the intergenic region between *orf66* and *kilB* when it encounters *kilB*-promoter-bound KorB molecules? In this chapter, we define further the events that occur in this region. Our results show that in addition to the transcription elongation complex apparently displacing KorB during readthrough transcription, operon transcription is also terminated here at some frequency. Furthermore, KorB appears to be a critical factor leading to termination, perhaps acting as a physical barrier to transcription. Our evidence also implicates that a hairpin located between the 3' end of *orf66* and the 5' end of the KorB binding site on the operon mRNA affects antitermination in opposition to KorB-mediated termination.

In addition to observing termination in the intergenic region, there was an interesting although logical correlation between readthrough transcription and the appearance of the truncated products. The pattern of readthrough transcription was consistent with previous results (Schully and Pettis 2003) that indicated readthrough transcripts increase throughout growth. Here, little to no readthrough transcription was observed during mid-exponential growth (Figure 3.2, lane 3), but it then became

noticeable at late exponential phase and persisted throughout stationary phase (Figure 3.2, lanes 4 and 5). When compared to the termination results, it becomes apparent that the lack of readthrough during early and mid exponential phase (Schully and Pettis 2003, this study) is the result of extremely efficient termination of the operon transcript occurring at a discrete point in the intergenic region (Figure 3.2, lane 3). As readthrough transcription becomes evident, the pattern of termination becomes more diffuse (Figure 3.2, lanes 4 and 5), perhaps an indication that termination is now less efficient.

Repression of transcription from the *kilB* promoter occurs via the binding of KorB repressor to the *kilB* operator sequence (Stein et al. 1989). Ducote and Pettis (2003) recently demonstrated that KorB protection extends from -40 to +21 relative to the *kilB* transcription start site (Figure 3.1). Because of the small size of KorB, ~6 kDa (Tai and Cohen 1993), these data suggest that numerous molecules of KorB are interacting at the *kilB* promoter, an effect that could promote its ability to function as an effective roadblock to elongating RNA polymerase molecules. Investigators have considered the possibility that DNA binding proteins could act as barriers to transcription dating back to the early studies of the *lac* operon (Reznikoff et al. 1969). Since then, the *lac* repressor has been used to facilitate transcription elongation arrest in order to study the transcription elongation complex in vivo (Arndt and Chamberlin 1990). Still other investigators have demonstrated termination in vitro using mutant DNA binding proteins (Pavco and Steege 1990, 1991), and a recent study has presented strong inferential data supporting a potential in vivo roadblock (Dole et al. 2004), although the unambiguous demonstration of a naturally occurring roadblock appears to remain elusive. Unfortunately, the lethal phenotype associated with unregulated *kilB* (Kendall and Cohen

1987) made it impossible to assess the role of KorB in transcription attenuation on a pIJ101 replicon, a complication that necessitated the use of artificial constructs. Quantitative data proved that pSCON93 is a suitable model for studying readthrough-dependent *kilB* expression. S1 mapping performed in the presence and absence of KorB demonstrated that termination of upstream-initiated transcripts on pSCON93 also occurs in close proximity to the known position of bound KorB repressor molecules. These data are consistent with KorB acting in a previously undefined role, a roadblock to elongating operon transcription. Although some termination was apparent in the absence of KorB, these products appeared only following prolonged exposure (Figure 3.3 C) and may indicate that KorB is not the sole terminating factor.

Traditional models for prokaryotic transcription termination begin with a pause site for the RNA polymerase (e.g., the formation of a stem loop in the nascent transcript in factor-independent termination) followed by destabilization of the complex resulting in termination and release either facilitated by Rho (i.e., for factor dependent termination) or the weak base pairing of the run of A:Us in the RNA:DNA hybrid (i.e., for factor independent termination). Because of high (73%) GC content of *Streptomyces* DNA, stretches of dA-residues are quite rare and thus, factor-independent terminators without poly-U tails have been documented in the genus *Streptomyces* (Deng et al. 1987). Although the IR element in the intergenic region separating *orf66* and *kilB* resembles such a terminator, and may play a role in the low levels of terminated transcripts seen in the absence of KorB for pSCON278 (Figure 3.3 C), our data are not consistent with the IR functioning in termination in its natural position on pIJ101. Instead the effects of deleting or altering the IR region downstream of *orf66* are consistent with a role for this



region in antitermination of operon transcription. This seems like a logical conclusion considering that in addition to the effects of KorB-mediated termination of operon transcription, the non-translated region of the readthrough transcript would be particularly vulnerable to transcription termination signals. Considering the necessity of readthrough to *kilB* expression (Schully and Pettis 2003), the existence of a mechanism that would allow the operon transcription elongation complex to ignore termination signals would therefore seem crucial in order for readthrough to be successful.

Although the actual mechanisms that confer upon RNA polymerase the ability to ignore transcription termination signals are largely misunderstood, antitermination in all manner of bacterial and bacteriophage operons is well documented (reviewed in Gollnick and Babitzke 2002, Stülke 2002, Weisberg and Gottesman 1999, Rutberg 1997, Friedman and Court 1995). Currently, there are no consistent models such as those that exist for termination, although many models for antitermination involve the formation of a stem-and-loop in the nascent transcript. In some models, the flanking sequences surrounding the hairpin recruit RNA binding proteins that take part in antitermination (Nodwell and Greenblatt 1993). Additionally, antitermination factors have been demonstrated to bind to the hairpin itself (Lazinski et al. 1989). Here, the effects of mutations introduced in and around the IR sequence between *orf66* and *kilB* demonstrate specificity for the sequence 5' to the hairpin as well as for the putative stem-and-loop itself. Although we cannot rule out other possibilities such as intrinsic antitermination facilitated by alternative structures forming in the readthrough transcript, our observations make an antitermination model involving additional putative RNA or protein factors (reviewed in Renan 1989, Gollnick and Babitzke 2002,) an attractive possibility. Additional, more

focused studies such as systematic mutagenesis of each nucleotide in and around the hairpin will be required to better define this intricate regulatory system. It would also be interesting to investigate the role of the host in antitermination (see chapter five for detail).

## **Materials and Methods**

### **Bacterial Strains and Plasmids**

*S. lividans* strains TK23, TK64 and 1326 have been described elsewhere (Kieser et al. 2000) as was the *Streptomyces* plasmid pHYG3 (Pettis and Cohen 1994). Propagation of plasmids was carried out in the *E. coli* host DH10B (Invitrogen). To construct the artificial model for readthrough-dependent expression of *kilB*, pPM927 (Smokvina et al. 1990) was digested with *Bam*HI, treated with Klenow and nucleotides to make the ends flush, and then finally digested with *Hind*III to produce two fragments of 0.65 and 2.0 kb. The resulting 0.65 *Bam*HI (blunt ended) – *Hind*III fragment containing the *tipA* promoter and bacteriophage fd terminator [*ter*(fd)] was cloned into the *Sma*I – *Hind*III sites of pSP72, producing pSCON73. The 2.0 kb *Hind*III fragment containing the spectinomycin/streptomycin cassette was then cloned into the *Hind*III site of pSCON73 and, following verification of the insert's orientation, this clone was designated pSCON75. pSCON93 was then constructed by inserting the 2.65 kb *Pvu*II – *Eco*RV fragment of pSCON75 into the blunt-ended *Hind*III site of pSCON61 (Ducote and Pettis 2003), which is pSCON2 (Schully and Pettis 2003, Ducote and Pettis 2003) carrying the *korB* gene. Inserting the fragment directly into pSCON2 at the blunt-ended *Hind*III site produced pSCON278.

Introduction of the *NsiI* site was carried out by two-cycle PCR as shown in Figure 3.5. Reaction 1 included as the template pGSP292, which contains the 1.0kb *PstI-EcoRI* of pHYG3-L41 (pHYG3 with an *EcoRI* linker inserted at the *BalI* site in *orf56*) (G. Pettis, unpublished results) cloned into those sites of pBluescriptSKII+ (Stratagene). Also include was the mutagenic primer *kilBMUT1*: 5'-GGGGCCATGCATCATGCGG (mutations in bold) and the T3 Promoter primer (NEB) specific for the T3 promoter contained on pBluescriptSKII+. Reaction 2 contained pGSP292, primer *kilBMUT4*: 5'-GCCTACCTCGCCACCGTGG and the T7 Universal primer (NEB) specific for the T7 promoter contained on pBluescriptSKII+. The resulting products were mixed, denatured and allowed to reanneal, thereby producing hybrids. Klenow fragment and nucleotides were added to produce double-stranded DNA, which was used as a template in a second round of PCR that contained the T3 and T7 primers discussed earlier. The resulting 1,137-bp product was then digested with *EcoRI* and *PstI* and cloned into those sites of pBluescriptIISK+. Clones were screened for *NsiI* digestion and sequenced to ensure only the desired mutations were present, and one of the clones that met these criteria was designated pSCON62. In order to delete the symmetrical region between *orf66* and *kilB*, pSCON62 was used as the template for a PCR reaction with primers *kilBNsiI*: 5'-AAAATGCATCCTACCCATTGCGAAACCTGAC and *kilB3*: 5'-TCAGGCGCCGAACCGGCGGGCGGCG. The 530-bp product was digested with *NsiI-BsiWI*, and the resulting 301-bp fragment was then cloned into pSCON62 at those same sites to produce pSCON154. pSCON154 was then used as a template for PCR using *kilBΔhp*: 5'-GTCAGGTTGCGCAATGGGTAGGCATCCGTCA TGCGGCGG and the SP6 Universal (NEB) vector primer complimentary to the SP6 promoter on

pSP72. Similarly, pGSP298 (2.7 kb *Pst*I-*Xho*I from pHYG3 in those sites of pSP72) (Matt Ducote, unpublished results) was used as a template for PCR using primers SP6 and *kilBMUThp*: 5-GTCAGGTTGCGCAATGGGTAGGCCCCGCCGGTTTCCTGC TGGT CCATCCGTCATGCGGCGG or *kilBMUThpX2*: 5'GTCAGGTTGCGCAATG GGTAGGACCAGCAGGTTT CCTGCTGGTCCATCCGTCATGCGGCGG. The resulting ~0.35 kb fragments were digested with *Fsp*I and *Pst*I, and the 281-bp fragments were cloned separately into those sites of pSCON279, which contained the *Pst*I-*Eco*RI fragment of pHYG3(L41). The 563-bp *Bsi*WI and *Pst*I fragments from each of the resulting plasmids pSCON280 (MUThp), pSCON281 (MUThpX2), pSCON282 ( $\Delta$ hp) and pSCON62 (*Nsi*I) were subcloned into pGSP298. Each of the pGSP298 derivative plasmids was then digested with *Pst*I and *Xho*I, and the 2.7-kb fragments were cloned separately into those sites of pGSP306. pHYG3 with each of the desired mutations was reconstituted by ligating the 6.1-kb *Bgl*II-*Xho*I fragments of each pGSP307 derivative to the 4.4 kb *Bgl*II-*Xho*I fragment of pHYG3. For S1 mapping probes, pGSP298, pSCON280, pSCON281, pSCON282 and pSCON62 were digested with *Bst*EII, the ends were made flush with Klenow fragment and nucleotides, and a *Pst*I digestion was then performed. The resulting 360-bp fragment was cloned into pSP72 at the *Xba*I (blunt-ended) and *Pst*I sites producing pSCON201, pSCON291, pSCON292, pSCON293 and pSCON294. All PCR reactions were carried out using Pfu polymerase (Stratagene), and the products were sequenced to ensure only the desired mutations were achieved before proceeding to subsequent steps.

## **Molecular Biology Techniques**

Molecular cloning was carried out as described by Sambrook et al. (1989), Western blotting for KilB protein was previously described by Pettis et al. (2001), and genetic manipulation of *Streptomyces* was described by Kieser et al. (2000). Induction of pSCON93 and pSCON278 was carried out as described in the text. Catechol dioxygenase activity was determined as previously described (Ingram et al. 1989, Schully and Pettis 2003). Assessment of conjugation was carried out as described in Pettis and Cohen (1994). Briefly, spores of *S. lividans* strain TK64 were spread onto non-selective R2 + 0.1% yeast extract plates (Kieser et al. 2000). The resulting lawn was then inoculated by pipetting 10 µl of donor pores in a line across the recipients. Following growth for one complete life cycle (7-10 days), replica plates were made on media selecting for transconjugants with Hygromycin and Streptomycin. Conjugation efficiency was assessed by measuring the distance from the center of the donor line to the edge of the transconjugants as compared to the wild type.

## **RNA Isolation and Analysis**

RNA was isolated by lysing the cells in modified Kirby mixture followed by organic extractions, and DNA was removed by enzymatic digestion all as described (Kieser et al. 2000). RNA preparations were analyzed by agarose gel electrophoresis to assess RNA integrity (Appendix B) and were subjected to PCR amplification using primers anchored in the intergenic region (*kilBMUT4* and *kilBxtn* (Schully and Pettis 2003) to assess DNA contamination. Any RNA preparation that produced an amplified product was subjected to a second treatment with DNase to ensure it was DNA free. RNase digestion of RNA samples prior to completion of S1 protocol was carried out as

described in Sambrook et al. (1989), and following organic extraction RNA was precipitated with 50 µg of carrier yeast-tRNA. S1 mapping was carried out as described (Kieser et al. 2000), except 50 µg of total RNA was hybridized with the probe. The probe for pHYG3, pSCON93 and pSCON278 analysis was a 231-nucleotide *SalI-EcoRV* fragment from pSCON201 that was filled in with Klenow in the presence of [ $\alpha$ -<sup>32</sup>P]dATP and [ $\alpha$ -<sup>32</sup>P]dCTP, along with the two remaining cold nucleotides. For intergenic mutant analysis, *SalI-EcoRV* fragments from pSCON291 (for pHYG3-MUThp), pSCON292 (for pHYG3-MUThpX2), pSCON293 (for pHYG3- $\Delta$ hp) or pSCON294 (for pHYG3-*NsiI*) were prepared as described above and hybridized to the appropriate RNA. Hybrids were subjected to S1 nuclease (Promega) digestion according to protocol specifications (Kieser et al. 2000). The resulting products were electrophoresed on an 8% denaturing Long Ranger (BioWhittaker Molecular Applications) polyacrylamide gel alongside the sequence of pSP72 (Stratagene), which was generated with the Sequenase (USB) sequencing kit and the T7 promoter primer. Analysis of predicted RNA secondary structure was performed using Mfold (Zuker 2003).

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**CHAPTER FOUR:**  
**EXPRESSION CHARACTERISTICS OF THE *STREPTOMYCES IPOMOEAE***  
**GROUP III INHIBITOR, IPOMICIN**

## Introduction

The antibiotic-producing soil-dwelling genus *Streptomyces* undergoes a complex cycle of morphological and physiological differentiation followed by periods of cryptobiotic dormancy protected by spores. Because of an extremely proficient system of conjugal horizontal gene transfer that apparently occurs through a novel mechanism (Possoz et al. 2001), the streptomycete genome is a continuum of diversity throughout the genus. This diversity is reflected in the utilization of an array of carbon sources, microbial antagonism involving, collectively, thousands of secondary metabolic compounds, and the albeit rare ability to cause disease (Keiser et al. 2000).

The spore of a *Streptomyces* species that encounters favorable growth conditions germinates and frequently branching hyphae emerge, producing a weave of intermingled mycelia. When cultivated on agar media, the resulting growth occurs along the surface of the medium as well as penetrating the substratum and thus is termed the substrate mycelium, which is characterized by infrequently spaced multigenomic compartments. Prolonged growth results in nutrient deprivation and the emergence of vertically directed aerial hyphae from the substrate. The aerial mycelia are typified by the colony taking on a fuzzy white appearance and by the production of secondary metabolites. Finally the aerial hyphae become coiled and unigenomic compartments differentiate into spores (Chater 1993). Under laboratory conditions, streptomycetes can also be propagated in liquid media where mycelia grow as interlaced bundles of mycelia which display a multi-phase growth curve similar to that of unicellular bacteria. While growing in liquid, *Streptomyces* bacteria readily undergo secondary metabolism as the culture enters

stationary phase, but they do not differentiate morphologically (Champness and Chater 1994).

The complex life style of streptomycetes allows for the isolation and characterization of mutants that define growth-phase regulators. *bld* (“bald”) mutants, for example, are a class of mutations observed to be deficient in morphological differentiation and antibiotic production (Merrick 1976, Champness 1988). The term “bald” refers to the fact that these mutants fail to produce aerial hyphae and therefore do not take on the fuzzy appearance typical of the aerial mycelium. The first to be discovered and probably most interesting of the *bld* mutants is *bldA*. Genetic analysis localized the *bldA* mutations to a proposed tRNA gene (Lawlor et al. 1987 and Chater et al. 1998), which was later proven to encode for the tRNA that recognizes the UUA (leucine) codon (Leskiw et al. 1991). Because of the high G-C content of *Streptomyces* DNA (approximately 73%), codons devoid of G or C residues are quite rare (Wright and Bibb 1992) and thus may be used to regulate translation of messages. To date UUA codons have not been found in genes that encode essential functions and deletion of the *bldA* gene had no effect on vegetative growth (Leskiw et al. 1991); UUA codons are however often found in positive regulators of antibiotic biosynthetic gene clusters. *bldA* makes an effective growth-phase regulator because although it is expressed throughout growth it appears to produce an inactive precursor. Accumulation of mature tRNA<sup>Leu</sup><sub>UUA</sub> occurs only as the culture ages, coincident with the late translation of TTA-containing genes (Leskiw et al. 1991). In surface grown cultures, translation of UUA-containing mRNAs coincides with aerial hyphae formation while in liquid cultures it occurs as the culture transitions into stationary phase.

The genus *Streptomyces* is comprised of greater than 500 species. Although the large majority of species are considered to be beneficial, in that they produce countless biologically active compounds such as antibiotics, a handful are also plant pathogens. Although much is known about the pathogenicity of Gram-negative counterpart genera such as *Pseudomonas*, *Xanthomonas* and *Xyella* (reviewed in Galan and Collmer 1999, Hueck 1998), like most other aspects of *Streptomyces* biology that do not involve secondary metabolism, pathogenic streptomycetes have received little attention through the years. However, as the number of pathogenic species identified increases and their economic impact grows, more and more investigators are turning their attention to pathogenic *Streptomyces* species.

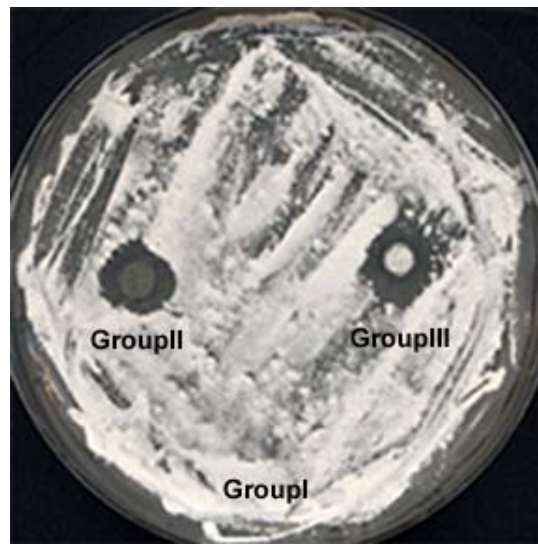
Once again distinguishing themselves from typical bacteria, plant pathogenic streptomycetes infect the roots of susceptible plants as opposed to aerial tissue such as leaves and fruit (reviewed in Loria et al. 2003, Galan and Collmer 1999, Hueck 1998). The molecular basis for streptomycete root invasion lies in the production of a unique class of nitrated dipeptide phytotoxins called thaxtomins (Lawrence et al. 1990, Healy et al. 2000). These compounds allow invasion of intact plant tissue (Clark and Matthews 1987), perhaps by compromising the integrity of the cell wall (Fry and Loria 2002), and thus streptomycete pathogens do not require a preexisting opening or wound for entry (Loria et al. 2003).

Pathogenic *Streptomyces* species are best known for the production of potato scab on potato tubers and other root crops including turnip, carrot, radish and even peanut (Loria et al. 1997). The disease is characterized by raised, rough and corky lesions on the storage roots of susceptible plants. The major etiological agent of potato scab is

*Streptomyces scabies* (Lambert and Loria 1989a). Distributed worldwide, *S. scabies* is the most economically important and the most widely investigated pathogenic streptomycete, and experimental evidence suggests that it is probably the original *Streptomyces* plant pathogen (Loria et al. 2003). *Streptomyces turgidiscabies* causes potato scab in Japan (Miayajima et al. 1998) and Europe, while *Streptomyces acidiscabies* (Lambert and Loria 1989b) is capable of infecting the same plants as *S. scabies* but at a lower soil pH (Loria et al. 1997). Both *S. turgidiscabies* and *S. acidiscabies* cause symptomatically indistinguishable diseases from *S. scabies* but the three organisms are genetically distinct (Loria et al. 2003). Invasion of root tissue by these bacteria is facilitated by thaxtomin A (Loria et al. 1997), and necrosis is the result of the *nec1* gene product (Bukhalid and Loria 1997). The infectious functions are all contained on a horizontally transferable pathogenicity island (PAI) (Bukhalid and Loria 1997, Kers et al. 2005) that was probably acquired from another taxon first by *S. scabies*, which subsequently transmitted it horizontally to *S. acidiscabies* and *S. turgidiscabies* (Kers et al. 2005, Healy et al. 1999, Bukhalid and Loria 1997).

Similarly, sweet potatoes are plagued by “soil rot” caused by *S. ipomoeae* (Clark and Moyer 1988). Soil rot is characterized by necrotic lesions on the storage roots of the sweet potato *Ipomoea batatas*, thereby reducing their market value; soil rot also destroys the feeder roots of sweet potato plants and in so doing reduces their yield of storage roots (Clark and Matthews 1987). Very little is known about *S. ipomoeae* pathogenicity but it is known to produce thaxtomin C as its major virulence factor (King et al. 1994), and it appears to lack the *nec1* necrosis factor (Bukhalid and Loria 1997).

Numerous strains of *S. ipomoeae* have been identified and divided into three groups based on their antagonistic characteristics. Termed interstrain inhibition, two of the three groups produce an inhibitor of members of the other groups (Clark et al 1998). Members of group I are inhibited by compounds produced by groups II and III (Figure 4.1), but are not capable of producing any inhibitory compound themselves (Clark et al. 1998). Group II strains inhibit groups I and III while group III strains produce an inhibitor of groups I and II (Clark et al. 1998).



**Figure 4.1. Example of interstrain inhibition by the *S. ipomoeae* inhibitors.** 5  $\mu$ l of spores of *S. ipomoeae* strains 88-35 (group II) and 91-03 (group III) were inoculated onto a lawn of *S. ipomoeae* strain 78-61 (group I) spores spread on *Streptomyces ipomoeae* growth agar (SIGA) (Clark and Lawrence 1981). Co-cultivation was continued until sporulation to demonstrate the inhibition of group I by groups II and III.

Previously, Zhang et al. (2003) purified the *S. ipomoeae* group III inhibitor. The inhibitor activity was directly attributed to a 10-kDa bacteriocin-like protein that is secreted into the medium, which was designated ipomicin. The N-terminal sequence of ipomicin was determined and utilized to construct a degenerate oligonucleotide, which was used to probe a *S. ipomoeae* strain 91-03 cosmid library. The structural gene,

designated *ipoA*, was located on the cosmid pSIP7 and its sequence was determined (Zhang et al. 2003). Additional sequence on the 5' end of the *ipoA* gene was identified, suggesting that *ipoA* is expressed as a 13 kDa precursor, which is processed to a 10-kDa secreted form. Interestingly, within the region off *ipoA* that encodes the signal sequence of ipomicin, a single TTA codon is present, which may signify a target for *bldA* regulation (Leskiw et al. 1991).

In this chapter, I will examine the *ipoA* locus and its promoter elements as well as begin to elucidate the expression characteristics of the *ipoA* gene and its gene product ipomicin. *ipoA* mRNA was found to originate from a promoter 56 bp upstream of the *ipoA* ORF. Transcription from the *ipoA* promoter appears to be temporally regulated in a manner that conflicts with the appearance of ipomicin in the medium, which dramatically increases as the culture transitions into stationary phase. Interestingly, susceptible strains of groups I and II also contain and express *ipoA*. The potential for *bldA* regulation of ipomicin is discussed.

## **Results**

### **Identification of the *ipoA* Promoter Elements**

The cosmid pSIP7 that Zhang et al. (2003) found to possess *ipoA* was sequenced (by Brenda Grau), and the sequence demonstrated that the *ipoA* locus contains an additional open reading frame (ORF) in the opposite orientation to *ipoA* (Figure 4.2 A), which is separated by 243 base pairs (Figure 4.2 B). This ORF BLASTs as a putative deoxyribonuclease found on the *Streptomyces coelicolor* chromosome (SCO2737), designated *recD*. To begin our understanding of *ipoA* expression, we sought to map the transcription start sites and identify the promoter elements within the intergenic region.



First, employing primer extension involving total RNA derived from *S. ipomoeae* strain 91-03, two reverse-transcribed products were generated by both the *recD*-specific primer (Figure 4.2 C) and the *ipoA*-specific primer (Figure 4.2 D). Using a 283 nucleotide (nt) 5'-end-labeled DNA probe complimentary to either the *recD* or *ipoA* message, we also probed for transcripts originating in the intergenic region by S1 nuclease protection assays. S1 probes were PCR-amplified products of reactions containing primers *ipoA*xtn and *recD*xtn (Figure 4.2 B), one of which had been previously labeled on the 5' end by using [ $\gamma$ - $^{32}$ P]-ATP and T4 polynucleotide kinase.

The S1 probes hybridized to mRNA derived from *S. ipomoeae* strain 91-03. The *recD* protected fragment (Figure 4.2 E, lane 1) migrated with the sequencing ladder at a position which was consistent with a doublet observed in one of the primer extension products (Figure 4.2 C, indicated with an asterisk) and indicates that *recD* transcription initiates at adenine and guanosine residues located 96 and 97 base pairs upstream of the *recD* translation start site, respectively (Figure 4.2 B). The *ipoA* probe protected a fragment 76 nt long corresponding to initiation at a guanosine residue 56 base pairs upstream of the *ipoA* start of translation (Figure 4.2 B) and appeared to have a second minor transcription start site one nucleotide larger (Figure 4.2 F, lane 2). The major transcription initiation site was consistent with one of the transcription start sites determined primer extension (Figure 4.2 D, indicated with an asterisk). Our analysis determined that *ipoA* and *recD* are expressed from divergent promoters that do not appear to overlap, a result that implies that the two cistrons may not be co-regulated at the transcriptional level.

**Figure 4.2. The *ipoA* locus of *S. ipomoeae* strain 91-03 and determination of its promoter elements.**

**A)** Physical map of the *ipoA* locus. The open reading frame (ORF) of *ipoA* and the putative ORF *recD* are indicated, along with their common promoter region.

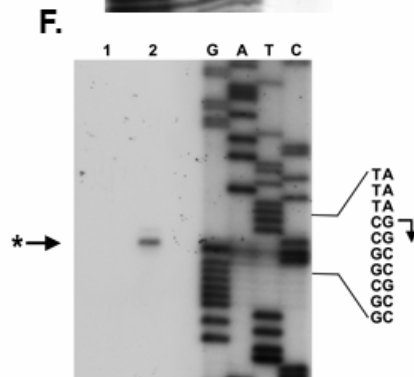
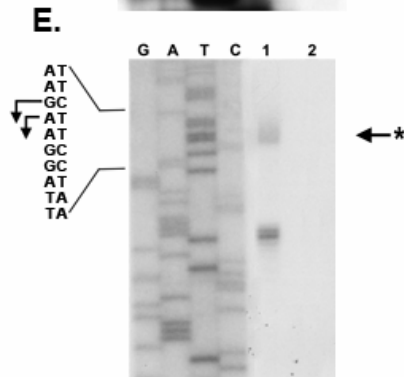
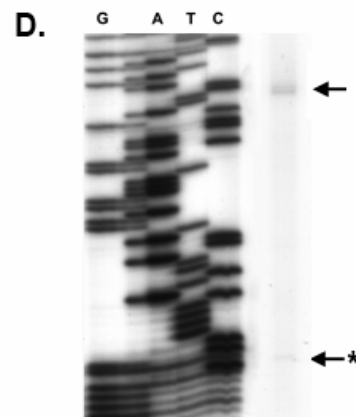
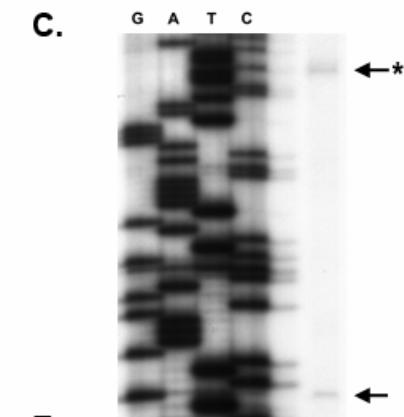
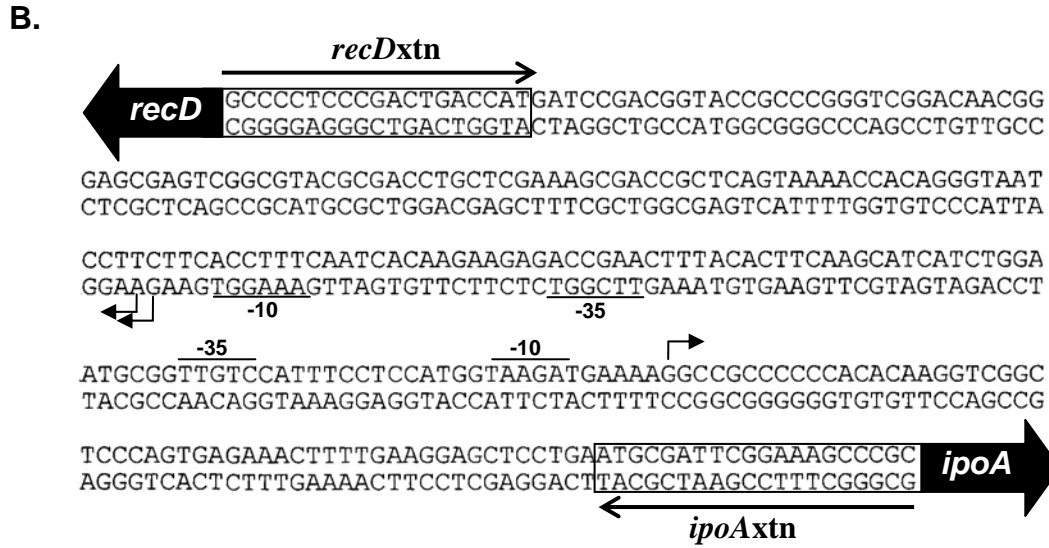
**B)** Sequence of the intergenic region between *ipoA* and *recD*. The divergent promoters with the major sites of transcription initiation are indicated by arrows and putative -10 and -35 promoter elements are also indicated for each promoter. The position and direction of primers *ipoA*xtn and *recD*xtn used in primer extension and S1 probe construction are also indicated.

**C)** Primer extension analysis of the *recD* transcription start site. Total RNA was isolated from *S. ipomoeae* strain 91-03 during exponential phase, then 10 µg was hybridized to the primer *recD*xtn depicted in part B, and primer extension was carried out as described in McDowall et al. (1994). The resulting cDNA was resolved on a 8% Long Ranger sequencing gel alongside the sequence of pSIP35, which was generated using the primer *recD*xtn. Two extension products were evident (indicated by arrows) - doublet (indicated with an asterisk) and a lower, single product - from RNA derived from *S. ipomoeae* strain 91-03 (lane 1).

**D)** Primer extension analysis of the *ipoA* transcription start site. 10 µg of the same RNA described in C was hybridized to the primer *ipoA*xtn depicted in part B, and primer extension was carried out as described above except that the sequencing ladder was generated using the primer *ipoA*xtn. Again, two products were evident in RNA derived from strain 91-03, indicated by arrows as shown in lane 1.

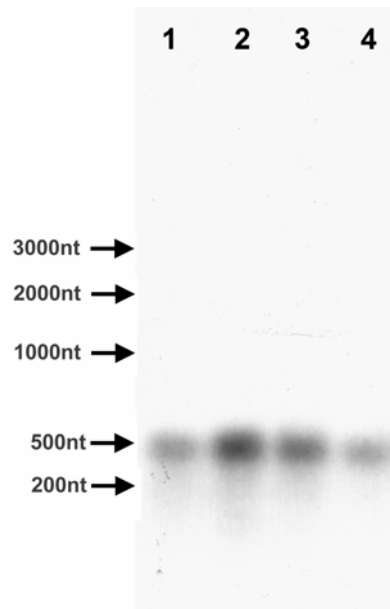
**E)** S1 nuclease protection of the *recD* transcription start site. Total RNA was isolated from cultures of either *S. ipomoeae* strain 91-03 (OD<sub>600</sub> = 0.684), or *S. lividans* strain TK23 (OD<sub>600</sub> = 0.8) and hybridized to a 5'-end labeled DNA probe that was generated by PCR using labeled *recD*xtn primer and cold *ipoA*xtn. Reactions were then subjected to S1 nuclease protection as described in Kieser et al (2000). Protected fragments were electrophoresed (lane 1- *S. ipomoeae* strain 91-03, lane 2 – *S. lividans* strain TK23) through a denaturing 8% polyacrylamide gel alongside the sequence of the intergenic region generated using pSIP35 as the template and *recD*xtn, and the gel was then subjected to autoradiography. Two fragments were protected by the probe, but only the larger one (indicated with an asterisk) corresponded to an extension product (i.e., the larger one marked with an asterisk) of the primer extension in part C.

**F)** S1 nuclease protection of the *ipoA* transcription start site. S1 mapping was conducted as described in E except that the probe was generated using labeled *ipoA*xtn and cold *recD*xtn. A single protected fragment was evident (marked with an asterisk) and corresponded to the larger of the two primer extension products in part D. Lane 1 contains the RNA isolated from *S. lividans* strain TK23 and lane 2 contains RNA isolated from *S. ipomoeae* strain 91-03.



### **The *ipoA* Gene Is Temporally Expressed**

To examine the expression pattern of *ipoA* temporally, we probed total RNA isolated from increasing culture densities of *S. ipomoeae* strain 91-03 for the *ipoA* message by Northern blotting using  $^{32}\text{P}$ -labelled fragments of the *ipoA* gene. The position of the *ipoA* promoter (Figure 4.2 B) suggests that if expressed as an individual entity, the *ipoA* mRNA would be ~450 nt. Northern blot analysis (Figure 4.3) revealed that the probe hybridized to a single message that migrated alongside the 500 nt band of the RNA ladder. Additionally, a temporal pattern of expression was observed, which indicated that the *ipoA* message was at its highest concentration in mid exponential phase (absorbance at 600nm = 0.684, lane2) and then declined at later timepoints reaching its lowest level late in stationary phase (absorbance at 600 nm= 2.081, lane 4).



**Figure 4.3. Northern blot for *ipoA* message from *S. ipomoeae* strain 91-03.** RNA was harvested from mycelia of *S. ipomoeae* strain 91-03 grown in liquid TSB to increasing culture densities ( $\text{OD}_{600} = 0.374, 0.684, 1.122$  and  $2.081$ , lanes 1-4 respectively). 30  $\mu\text{g}$  of total RNA were electrophoresed and subjected to Northern blot analysis as described by Vöggtli and Cohen (1992) using  $^{32}\text{P}$ -labelled fragments of the *ipoA* ORF generated by random priming as the probe. The positions of RNA size standards are indicated.

### **Ipomicin Production Is Temporally Produced in a Pattern Contrary to *ipoA* Transcription**

We sought to determine whether a correlation exists between *ipoA* expression and ipomicin production during growth. *S. ipomoeae* strain 91-03 spores were inoculated into liquid culture and incubated at 30°C while shaking. Samples were frequently harvested for the isolation of cell-free extracts of the supernatant and subsequent inhibitor quantitation (Figure 4.4 A), as well as to construct a growth curve (Figure 4.4 B). Ten-fold serial dilutions were performed on filtered supernatants, and 5.0 µl aliquots of the culture supernatants and dilutions were spotted onto test lawns of a susceptible strain (group II, strain 88-35). Activity was determined by measuring the zones of inhibition produced following incubation for three days at 30° C. A zone of inhibition of < 5mm was determined to contain “trace” amounts of inhibitor. For undiluted supernatants, inhibited zones of 5mm or greater (Figure 4.4 A, 25h and 27.5h) were designated 1U (per 5 µl, 200U/ml) while a 10-fold dilution that produced a zone of at least 5mm (Figure 4.4 A, 40h and 43.5h) was designated 10U (per 5 µl, 2000U/ml).

Results of our analysis determined that trace amounts of ipomicin became evident in the culture supernatant as early as 16 h post inoculation. By mid-exponential phase (Figure 4.4 B, 24hrs), the concentration reached 200U/ml (Figure 4.4 C) where it remained throughout the remainder of exponential phase. As the culture transitioned into stationary phase (Figure 4.4 B, 40hrs), ipomicin concentrations suddenly and dramatically increased 10-fold (Figure 4.4 C, 40hrs). The steady state concentration of ipomicin remained at 2000 U/ml for the duration of the study.

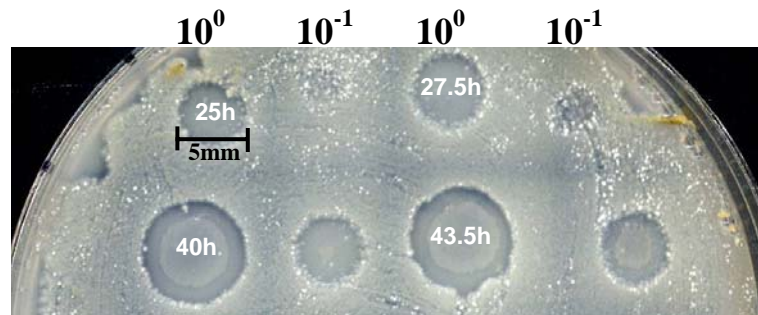
**Figure 4.4. Temporal release of *S. ipomoeae* group III inhibitor.**

**A)** Example of plate bioassay for the determination of ipomicin concentration. Cell-free extracts were made from culture supernatants as described in the Materials and Methods. 5  $\mu$ l of undiluted extracts or 10-fold serial dilutions were spotted onto SIGA inoculated with a lawn of *S. ipomoeae* strain 88-35 (group II) spores. Following incubation, the concentration of ipomicin was determined as described in the text.

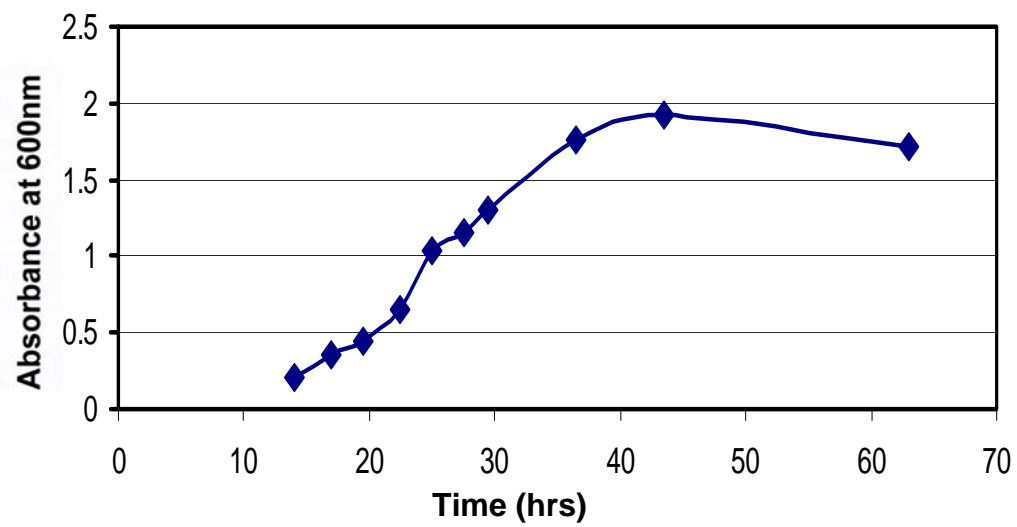
**B)** Growth curve of *S. ipomoeae* strain 91-03. Spores of *S. ipomoeae* strain 91-03 were inoculated into one liter of liquid TSB and incubated at 30° C while shaking. At frequent points throughout growth, mycelia were harvested and the culture absorbance was determined at 600 nm, the results of which were plotted as a function of time. The data shown are the average of three independent trials.

**C)** Production of group III inhibitor. At the same times as the samples were harvested for construction of the growth curve (B), additional samples were harvested and used to produce cell-free extracts of the supernatants which were subjected to the plate bioassay described in (A). The data shown are the average of the same three independent trials shown in (B).

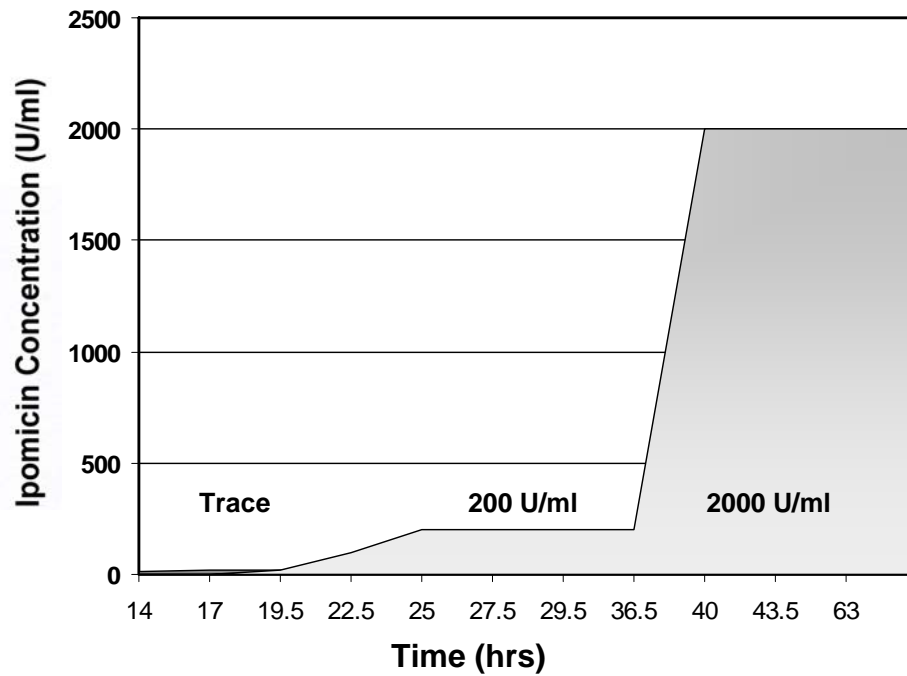
A.



B.



C.



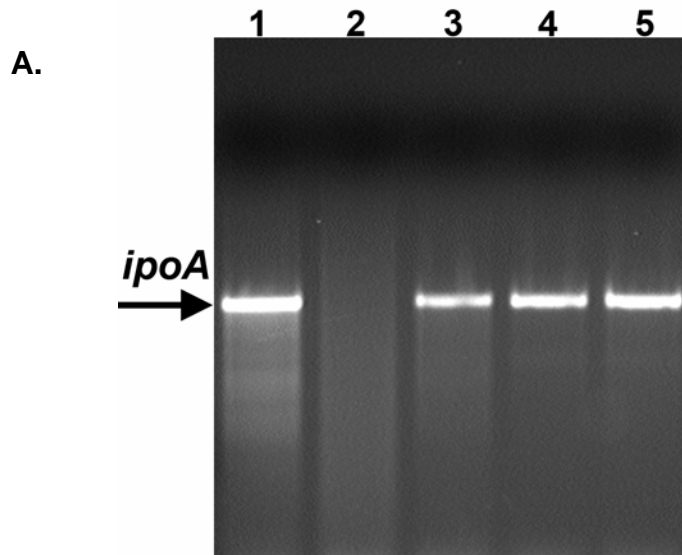
### **All Three Groups of *S. ipomoeae* Express the Wild Type *ipoA* Gene**

Because the three groups of *S. ipomoeae* are strains of the same species, we wondered if members of the ipomicin-sensitive groups I and II also contained the *ipoA* gene. If so, due to the toxicity of the *ipoA* gene product to groups II and III, it seemed logical that these genes would contain some defect that rendered them or their products dysfunctional in the susceptible strains. PCR using primers specific for the 3' and 5' ends of the *ipoA* ORF was conducted on genomic DNA isolated from representative strains of each group (i.e., strain 78-61 for group I, strain 88-35 for group II and strain 91-03 for group III). The data confirm that all three strains do in fact contain the *ipoA* gene (Figure 4.5 A). Furthermore, PCR-amplified products of all three groups migrated together (Figure 4.5 A) and appeared to be the same size indicating that there were no obvious deletions or insertions in the susceptible strains. Sequencing of *ipoA* from groups I and II revealed no mutations in either the structural gene or promoter region (data not shown). To confirm expression of *ipoA* in group I and II strains, Northern blot analysis on RNA isolated from cultures of strain 78-61 and 88-35 was conducted as described earlier for strain 91-03. This analysis demonstrated that *ipoA* is transcribed at least as well in these group I and II representatives as it is in strain 91-03 (Figure 4.5 B).

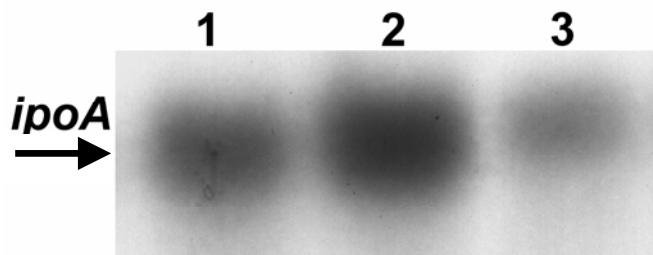
### **Discussion**

The pattern of ipomicin production for *S. ipomoeae* strain 91-03 contrasted greatly with the pattern of *ipoA* mRNA expression (compare Figure 4.3 to Figure 4.4, B and C). *ipoA* transcription was found at its highest concentration in early- to mid-exponential phases of growth followed by a gradual decline in amount transcription throughout the remainder of the study. This is an interesting observation considering





B.



**Figure 4.5. Members of all three inhibition groups of *S. ipomoeae* express the *ipoA* gene.**

**A)** PCR amplification of the *ipoA* ORF. Plasmid DNA pSIP7 (lane 1) and genomic DNA of *S. coelicolor* strain M145 (lane 2), *S. ipomoeae* strain 78-61 (group I, lane 3), strain 88-35 (group II, lane 4) and strain 91-03 (group III, lane 5) were used as the template for PCR involving primers *ipoA*5' and *ipoA*3' specific for the *ipoA* ORF. The resulting products were electrophoresed on a 1% agarose gel containing ethidium bromide, visualized under UV light and a digital image was recorded using an Eagle Eye II still video system (Stratagene).

**B)** Northern Blot analysis of RNA isolated from representative strains of all three groups of *S. ipomoeae*. RNA from *S. ipomoeae* strain 78-61 (lane 1), strain 88-35 (lane 2) and strain 91-03 (lane 3) harvested at similar culture densities during exponential phase were subjected to Northern blot analysis for the *ipoA* gene as described in Figure 4.3.

that ipomicin was observed only in trace amounts when *ipoA* transcription was increasing to its highest concentration, and was then found at 200 U/ml during the decline of *ipoA* transcription. What's more is the almost instantaneous ten-fold increase in ipomicin production as the culture entered stationary phase, where *ipoA* mRNA was found at its lowest concentration. How can ipomicin persist at its lowest concentration while the *ipoA* transcript is at its highest, and then dramatically increase in the presence of decreasing *ipoA* transcript concentration? The inverse relationship between *ipoA* transcription and ipomicin production could be the result of *bldA* regulation (Merrick 1976, Champness 1988, Leskiw et al. 1991, Leskiw et al. 1993). As noted earlier, the *ipoA* transcript contains a UUA (leucine) codon in the leader sequence (Zhang et al. 2003). The presence of this rare codon in the signal sequence presents an opportunity for *bldA* regulation of ipomicin. Although *bldA* codons are typically found in positive regulators of secondary metabolites, the presence of a target for *bldA* regulation in the signal sequence of a secreted protein would present a particularly powerful means of coordinating ipomicin production with *Streptomyces* growth phase (Keith Chater, personal communication). As for the presence of ipomicin during exponential phase (i.e., prior to *bldA* processing), early less efficient translation of UUA-containing mRNAs has been well documented and is proposed to be the result of inefficient translation by non-cognate tRNAs (Leskiw et al. 1991).

How can *bldA* regulation of ipomicin production be proven? Two experiments which are based on traditional methods for studying *bldA* regulation could be conducted in concert with each producing complementary data. First, mutation of the TTA codon in *ipoA* to a more common *Streptomyces* leucine codon (CTC for example) (Leskiw et al.

1991) followed by the plate bioassay described earlier would indicate whether there is now increased production of the ipomicin gene product prior to the appearance of mature tRNA<sup>Leu</sup><sub>TTA</sub>; in other words, if ipomicin is indeed the product of a *bldA*-regulated gene then it should now be present at high concentrations throughout growth. Similarly, numerous *bldA* mutants of other *Streptomyces* have been described, and so it is conceivable that an analogous *S. ipomoeae bldA* mutant could be isolated. When expressed in a *bldA* background, ipomicin should be continuously present at low levels indicative of inefficient translation and so would never produce the dramatic increase coincident with *bldA* processing.

These methods are tried and true. However, *S. ipomoeae* has proven to be extremely obstinate with regard to genetic manipulation. Chemical transformation, electroporation and conjugation have all been attempted many times by a number of personnel in our laboratory using numerous protocols, all to no avail. The genetic intractability of *S. ipomoeae* has made the methods traditionally utilized to confirm *bldA* regulation in the natural host impossible (for now). As a result, we will attempt to identify plasmid-borne ipomicin by Western blot. Polyclonal antibodies were recently raised against an ipomicin fusion protein (Schully and Pettis, unpublished data). The TTA codon in *ipoA* will be converted to a CTC codon using the two-cycle PCR procedure described in chapter 3, the product will be cloned onto a streptomycete plasmid, and the recombinant plasmid will be transformed into the isogenic pair of *S. coelicolor* strains M600 and M600  $\Delta bldA$  (kindly provided by Keith Chater). Ipomicin production will be monitored over time by Western blot from wild type (TTA) and mutant (CTC) *ipoA* sequences in both wild type and *bldA* mutant genetic backgrounds.

Although we cannot say with absolute certainty that the TTA in ipomicin represents a target for *bldA* regulation until these experiments are complete, our observations thus far, especially when considered with *Streptomyces* “dogma”, strongly support the notion.

Although the presence of the *ipoA* gene in all three groups was not unexpected, the lack of mutations in group I and II *ipoA* copies and the transcription of these genes in these susceptible strains is somewhat surprising. There is also no obvious basis for *ipoA* mRNA to not be translated in these strains as well. Because of the toxicity of ipomicin to groups I and II, it is clear that active inhibitor is not produced in these strains. It is our hope that the antiserum discussed earlier will aid in our understanding of the differences in ipomicin production between susceptible (group I and II) and resistant (group III) strains. It is possible that groups I and II lack the processing and transport functions required for the secretion of ipomicin and unprocessed inhibitor is tolerated by groups I and II until it is degraded. The accumulation of 13-kDa ipomicin precursor, as determined by Western blot, in groups I and II but not in III could signify the absence of the processing and secretion functions. It is also possible that further modification of ipomicin occurs in group III to produce the bacteriolytic effect and that such additional modification could effect the migration of active ipomicin as detected in Western blotting when compared to a putative inactive mature ipomicin form. It will be interesting to continue to study the production of ipomicin and to determine the role of *ipoA* in susceptible strains.

## **Materials and Methods**

### **Bacterial Strains and Plasmids**

All experiments were conducted in wild type *S. ipomoeae* strains 78-61 (group I), 88-35 (group II) and 91-03 (group III). pSIP7 was described in Zhang et al. (2003), and pSIP35 was constructed by PCR amplifying the intergenic region of the *ipoA* locus from pSIP7 using PFU polymerase (Stratgene) with the primers *ipoA*xtn: 5'-GCGGGCTTTCCGAATCGAT and *recD*xtn: 5'-GCCCCTCCCGACTGACCATG. The resulting 283 bp product was cloned into *EcoRV* site of pSTBlue-1 using the Perfectly Blunt PCR cloning kit (Novagen). The *E. coli* host for cloning was DH10B (Life Technologies Inc.).

### **Bacteriological and Molecular Biology Techniques**

Molecular cloning was carried out as described in Sambrook et al. (1989). Genomic DNA was isolated using the method of Rainey et al. (1996). Spores of *S. ipomoeae* were maintained on *Streptomyces ipomoeae* growth agar (SIGA) (Clark and Lawrence 1981), and liquid cultures were grown in Tryptic Soy Broth (TSB) (Sambrook et al. 1989). PCR was carried out as described in Pettis et al. (2001) using PFU polymerase (Stratagene) for cloning and GoTaq (Promega) for identification of *ipoA*. Manual sequencing was carried out with the Sequenase sequencing kit (USB). Sequencing reactions contained either *ipoA*xtn or *recD*xtn with pSIP35 as the template and  $\alpha$ -[<sup>32</sup>P]dATP (Amersham).

### **RNA Methods**

RNA was purified by first isolating total nucleic acid from the mycelia with glass beads in modified Kirby mixture, followed by enzymatic digestion of the DNA as

described in Kieser et al. (2000). Northern Blot analysis was carried based on Vöggtli and Cohen (1992); in this case, 30 µg of total RNA were resolved on a 1% agarose gel containing formaldehyde, blotted to a Hybond N (Amersham Biosciences) nylon membrane using the VacuGene XL vacuum blotting system (Amersham Biosciences) and hybridized to approximately  $1 \times 10^6$  cpm of probe as described elsewhere (Brasch et al. 1993). The probes were generated by random priming of a PCR-amplified template of the entire *ipoA* ORF with the NEBlot kit (New England Biolabs) and  $\alpha$ -[ $^{32}$ P]dCTP (6000 Ci/mmol) (Amersham Biosciences). PCR was carried out using PFU (Stratagene) polymerase and the primers *ipoA5'*: 5'-AAAAAGGATCCACAAGGTCGGCTCCCAGTGAG and *ipoA3'*: 5'-AAAAAGATCTCTGTCGCTCAGACGCGCAGG.

S1 mapping of the *ipoA* and *recD* promoters was carried out as described in Kieser et al. (2000), except that 10 µg of total RNA was hybridized to a 5'-end-labeled DNA probe. The probe was generated by first exchanging the  $\gamma$ -phosphate of either *ipoA*xtn or *recD*xtn (described above) with  $\gamma$ -[ $^{32}$ P]dATP (5,000 Ci/mmol) (Amersham Biosciences). The resulting 5-end labeled primers were used in a PCR reaction in conjunction with the opposite cold primer and pSIP35 as the template. The resulting 283-base pair PCR product was electrophoresed on a 1% agarose gel, and the probe was excised and purified from the agarose using a Qiaquick gel extraction kit (Qiagen).  $10^4$  cpm of the resulting 5'-end-labeled probe was hybridized to total RNA isolated from *S. ipomoeae* strain 91-03 during exponential phase. Following S1 nuclease digestion, the products were resolved on a 6% LongRanger polyacrylamide gel containing 8M urea alongside the sequence of pSIP35 which was generated using the Sequenase sequencing

kit (USB) and either *ipoA*xtn or *recD*xtn primers. Sequencing gels were dried and autoradiography was carried out at -80° C.

### **Plate Bioassay**

Ipomycin concentration was determined as previously described (Zhang et al. 2003). Briefly, spores of *S. ipomoeae* strain 91-03 were inoculated into one liter of liquid TSB and incubated at 30° C while shaking. One ml of culture was harvested at various points throughout growth, and the mycelia were removed first by centrifugation and then by filtration through a 0.2 µm filter, thereby producing a cell-free extract. Filtered culture supernatants were subjected to 10-fold serial dilutions in 50 mm sodium phosphate buffer (pH 7.5). 5 µl of both diluted and undiluted culture supernatants were spotted onto SIGA inoculated with a lawn of group II *S. ipomoeae* spores (strain 88-35). Following incubation, the resulting zones of inhibition were measured. One unit was previously defined as the highest 10-fold dilution that produced a zone of inhibition of at least 5 mm in diameter (Zhang et al. 2003). Therefore, a zone of inhibition derived from undiluted culture supernatant  $\geq 5$ mm was designated 1U (per 5µl, 200 U/ml), while a  $\geq 5$ mm zone derived from a 10-fold dilution was designated 10U (per 5µl, 2000 U/ml).

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**CHAPTER FIVE:**  
**CONCLUDING REMARKS AND FUTURE DIRECTIONS**

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The economic returns and indispensable importance to industry, agriculture and medicine has resulted in an overwhelming majority of the work conducted in *Streptomyces* biology to revolve around secondary metabolism, and the products that result. The goal of this work was to shed light on areas of streptomycete biology that have received comparatively little attention. Conjugal genetic exchange for example is well documented and assumed to be ubiquitous throughout the genus. Although investigators have acquired enough information to appreciate that conjugation in *Streptomyces* most likely represents a second paradigm in conjugal horizontal gene transfer, the actual mechanisms that govern streptomycete conjugation remain largely misunderstood. Pivotal discoveries in the field such as the identification of the first conjugative plasmid, the giant linear plasmid SCP1 (Vivian 1971), the discovery of the small circular plasmid pIJ101 (Kieser et al 1982) and the identification of a *cis*-acting locus involved in plasmid transfer (Pettis and Cohen 1994), just to name a few, have driven the field forward. However, our knowledge of the actual streptomycete conjugal mechanisms lags far behind that of their Gram-negative counterparts; for example, only recently has experimental evidence been presented inferring the double-stranded mode of streptomycete conjugation (Ducote et al. 2000, Possoz et al. 2001). Additionally, because of the very few plasmid-encoded functions involved in *Streptomyces* conjugation, it has long been speculated that numerous host-encoded functions must be involved; to date however, no such host-encoded conjugal genes have been identified.

My part of the story begins with the observation of the increasing pattern of expression displayed by the pIJ101 KilB spread protein (Pettis et al. 2001) and culminates with this work. Our observation that KilB increases throughout cellular

differentiation (Pettis et al. 2001) led to the investigation of the mechanisms that govern the temporal expression or accumulation of KilB protein. We found that the *kilB* gene, once thought to be separate from the upstream operon, is in fact expressed as part of the transmission operon. Moreover, in spite of the fact that *kilB* has its own promoter, operon transcription is required to initiate KilB protein expression on pIJ101. Regulation of the *kilB* gene, once thought to be dictated by its own negatively regulated promoter, is accomplished through the collaboration of numerous transcriptional and post-transcriptional control mechanisms resulting in the tightly controlled expression of *kilB*.

In chapter two, a model for transmission operon-readthrough-dependent *kilB* expression began to come to light. In spite of the fact that *kilB* has its own promoter, which has previously been shown by numerous methods to be active in the absence of KorB protein (Kendall and Cohen 1987, Buttner and Brown 1987, Pettis et al 2001, Schully and Pettis 2003), it appears that the natural state for that promoter in the presence of KorB (as on pIJ101) is to be largely, if not completely, repressed. Transcription from the upstream transmission operon then reads through the *kilB* operator region and is in fact required to trigger full expression downstream of the KorB-repressed-*kilB* promoter. Additionally, the data in Figure 2.6 demonstrates that transcripts are originating from the *kilB* promoter. One interpretation of the data presented in chapter two is that readthrough transcription results in the displacement of KorB repressor protein, allowing initiation to occur from the *kilB* promoter. Additionally, results presented in chapter three demonstrate, in vivo, KorB-mediated termination of operon transcription.

It would be interesting to examine the efficacy of the KorB roadblock, as well as the frequency of readthrough, in vitro. Protocols employed by Pavco and Steege (1990,

1991) would be applicable to assess the proposed KorB roadblock in vitro. In their work, the authors utilized a mutant *EcoRI*, which was capable of binding the *EcoRI* recognition sequence with high affinity without cleaving the substrate, and assessed its ability to block transcription in vitro by a variety of RNA polymerases. Here, purified processed KorB could be bound to a DNA template containing the *kilB* promoter sequence as well as an upstream promoter. *Streptomyces* RNA polymerase would then be added and in vitro runoff transcription would be carried out in the presence of [ $\alpha$ -<sup>32</sup>P]-CTP and other cold nucleotides (see appendix A, Figure A.1 for an example of in vitro runoff transcription). Following resolution of the products and autoradiography, runoff transcripts and truncated transcripts could be differentiated.

One critical aspect of the assay described above would be to ensure that each molecule of the template DNA was occupied by KorB repressor. This would ensure that the presence of full length runoff transcripts would be accurately interpreted as bypassing KorB and not simply the result of the template lacking KorB. However, attempts to purify large quantities of KorB to homogeneity for in vitro transcription assays met with considerable resistance. Tai and Cohen (1993) observed that KorB is expressed as a 10-kDa protein that is processed from the C-terminus into its 6-kDa active form. Furthermore, they observed complete processing occurs only in *Streptomyces*. As a result, overexpression of KorB in *E. coli* was not appropriate, and so we attempted to purify native KorB from *S. lividans* (see Appendix A, materials and methods for details). Following Tai and Cohen's purification protocol (1993) along with an inducible expression system for KorB protein (Appendix A, Figure A.2), we produced quantities only visible by Western blot (data not shown). I therefore attempted to utilize size

exclusion chromatography in an effort to take advantage of the small size of KorB. Interestingly, KorB eluted from the column in the fractions immediately following the void volume (Appendix A, Figure A.3), a result that would be expected from a protein considerably larger than 6-kDa.

It was possible that the gel filtration result was due to some type of oligomerization of KorB as it migrated through the column. I therefore attempted to denature KorB by incubating KorB-containing fractions from a heparin column in 8M urea at 37° C, and then separating the KorB monomers from contaminating proteins by forcing the solution through an Amicon YM10 (10-kDa molecular weight cut off) ultra filtration device. The urea was dialyzed away and the solution was concentrated with an Amicon YM3 filter (3-kDa molecular weight cut off). When the resulting concentrated fractions were analyzed by Western blot, KorB monomer was the predominant species (Appendix A, Figure A.4), however, additional, significantly larger protein bands were also apparent in the filtrate. Because theoretically only proteins smaller than 10-kDa would be present in the YM10 filtrate, one interpretation of these observations is that KorB has such a high affinity for itself that it is capable of maintaining multimeric species, even when electrophoresed through a denaturing polyacrylamide gel. Nevertheless, quantities of purified KorB remained at levels low enough to require visualization by Western blot. Moreover, electrophoretic mobility shift assays (EMSA) demonstrated that the quantities of purified KorB obtained thus far were not sufficient to completely shift the quantity of template that in vitro transcription would require (Appendix A, Figure A.5). While our goals of purifying KorB and then characterizing the KorB roadblock in vitro were not met, our observations suggest that KorB has a very

high affinity for itself and may therefore be capable of oligomerizing. This notion is consistent with Ducote and Pettis' (2003) observation of the surprisingly large footprint that KorB leaves on the *kilB* promoter, and could account for its ability to hinder readthrough transcription.

Although models suggesting transcription blockades imposed by DNA binding proteins are present in the literature (Renan 1989, He and Zalkin 1992, Dole et al. 2004), to my knowledge, no investigator has definitively proven a transcription roadblock. It was unfortunate that I was not able to unambiguously establish whether KorB is the sole termination factor. Due to the lethality of unregulated *kilB*, artificial constructs (pSCON93 and pSCON278) were necessary to investigate the termination potential of the readthrough transcript in the absence of KorB. The minor termination products observed for pSCON278 (Figure 3.3 C) may be the result of additional termination factors (He and Zalkin 1992) that can act at some frequency even in the absence of KorB or of some intrinsic termination occurring as a result of the IR. However, since pSCON278 does not contain the full transmission operon and expression is driven by *tipAp* rather than the *tra* promoter, the presence of low levels of termination in the absence of KorB may be irrelevant to what normally occurs on pIJ101.

In order to more accurately define the role of KorB in pIJ101 transmission operon attenuation, an experimental protocol that includes KorB will be necessary. One can imagine two possibilities that may alter the KorB roadblock without affecting the necessary repressor capability of KorB. One scenario involves mutation of the *kilB* operator sequence to affect the ability of KorB to bind to the *kilB* promoter. Although these mutations may also reduce *kilB* promoter activity, the S1 probe was designed not to



detect *kilBp*-initiated transcripts (Figure 3.2 A), and so readthrough versus termination for relevant mutants could still be monitored by the S1 procedure described in chapter three. It would also be interesting to mutate the *korB* gene in an effort to obtain KorB molecules that can bind the *kilBp* and repress transcription initiation, but perhaps not oligomerize as efficiently and block readthrough. Both *kilB* operator mutants and KorB repressor mutants could be identified using pSCON93 derivatives. Desired mutants would still produce ~3 mU/mg of XylE in the uninduced state (Figure 3.3 B), however, induction should increase XylE activity greater than 5-fold (Figure 3.3 B) due to the increased readthrough that would result from the mutation. It is also possible that mutations in *korB* could result in KorB molecules that do not allow readthrough. Once identified, the mutants would be cloned onto a pIJ101 derivative and the S1 experiments would be repeated.

Both the *c-myc* oncogene and the human immunodeficiency virus (HIV-1) are believed to contain transcription units regulated by a transcription roadblock, which is relieved by the association of antitermination factors with a stem-and-loop structure formed in the mRNA immediately prior to the blockade (reviewed in Renan 1989). The results depicted in chapter three demonstrate that in addition to attenuation of the operon transcript, antitermination of operon transcription is also occurring. Mutations introduced within and around the IR located immediately downstream of *orf66* (Figure 3.1) abolish operon readthrough transcription (Figure 3.6 B) and, consequently, KilB protein expression (Figure 3.6 A). The same effect was observed whether the IR was deleted entirely, or was mutated so that it contained mismatches in the proposed stem-and-loop structure, effects which both reduce the likelihood that the hairpin would form and

supported a model for secondary structure formation. Additionally, mutations that compensated for the aforementioned mismatches, and thus restored the hairpin's propensity to form, as well as mutations in the region immediately prior to the IR region which are not predicted to affect hairpin formation, had a similar effect on KilB production, suggesting sequence specificity for both the stem and the flanking sequence. These results are consistent with a model involving the IR region of the readthrough mRNA forming a stem-and-loop structure which functions in antitermination of readthrough transcription, possibly through the recruitment of transcription antitermination factors.

What is the role of the host in antitermination of operon transcription? Early genetic analysis of pIJ101 in which extensive insertion mutagenesis identified the regions of pIJ101 necessary for transfer and spread (Kendall and Cohen 1987) would have likely identified plasmid-borne genes encoding for antitermination factors, as these would have also behaved as spread functions due their positive effect on KilB expression. It therefore seems logical that host-encoded antitermination factors, and not plasmid-borne functions, may be acting to affect antitermination of operon transcripts. Although our previous studies have demonstrated that KilB expression is not directly tied to morphological cues (Pettis et al 2001), the conspicuous pattern of termination and readthrough observed in chapter two could indicate that putative antitermination factors are under the control of the *Streptomyces* global regulatory networks, *bld* or *whi* (reviewed in Chater 1993, 1998). The *bld* mutants are a class of mutations that are defective in aerial hyphae development and therefore do not take on the fuzzy white appearance indicative of aerial hyphae development (see chapter 4 for detail). Similarly,

*whi* (for white) mutants do not sporulate and thus do not take on the grey pigment associated with sporulation. To begin to investigate the role of growth-phase regulation in antitermination of operon transcription, we will examine the pattern and extent of transmission operon readthrough and attenuation in a *bldA* mutant background. These experiments are currently underway in an isogenic pair of *Streptomyces coelicolor*, strain M600 and M600 $\Delta$ *bldA* (provided by Keith Chater).

It has always been a goal of this study to quantitatively determine the frequency of the events described herein. For example, the ribonuclease protection assay (RPA) described in chapter two was originally designed to determine, in addition to the confirmation of readthrough, the relative contribution of each promoter to *kilB* expression (i.e., readthrough transcription as compared to the *kilBp*-initiated transcripts). However, because of readthrough, and the breakdown products that resulted, significant background was observed in RPAs performed on pIJ101-containing mycelia (see chapter two for detail) and quantitative interpretation of the results was impossible. When operon transcript termination was added to the model, quantitation of these events became a goal once more. For this, I attempted real-time PCR (rtPCR) utilizing SYBR Green (Applied Biosystems). By employing primer sets that would amplify regions exclusively in *orf66* or *kilB*, we could determine the amount of operon transcription, as well as the total amount of *kilB* transcript that was present. Also, utilizing a primer set which contained one primer anchored in *kilB*, and the other anchored in the intergenic region, we could determine the amount of readthrough that was occurring. These primer sets would be utilized in rtPCR with known amounts of the transmission operon to generate a standard curve, as well as with cDNA generated from RNA isolated from plasmid-containing

cultures. Finally, by comparing the values obtained from each primer set using the cDNA as the template to the corresponding standard curve for each primer set, it would be possible to determine the relative frequency of operon termination compared to readthrough, as well as to determine the relative contribution of readthrough to total *kilB* transcription. Although these experiments have not yet proceeded past the troubleshooting phase, eventual quantitative determination of the frequency of the expression events that occur within the intergenic region of the pIJ101 transmission operon would be a worthwhile aim of that project.

Models suggesting regulatory mechanisms that involve a repressor protein acting as a physical barrier to transcription elongation are growing (Dole et al. 2004, He and Zalkin 1992, Renan 1989). Data presented in this work support a model (Figure 5.1) suggesting the addition of KorB to that list. It would be interesting to continue to examine the role of KorB in pIJ101 transmission operon attenuation and unambiguously establish KorB as the pIJ101 transmission operon attenuator, as well as to further define the mechanisms that govern antitermination of this system. In conclusion, the KilB spread protein of pIJ101 presents an interesting paradox: a protein that is essential for efficient plasmid transmission, but is lethal to the host (Kendall and Cohen 1987). The solution to this problem was the evolution of an extremely intricate regulatory mechanism that allows for tolerable, yet functional levels of KilB protein to be produced; as to what the essential spread function of KilB is, however, remains to be determined.

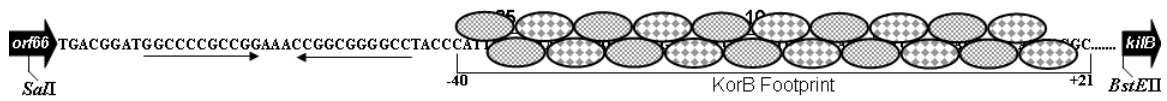
**Figure 5.1. A model for readthrough-dependent expression of *kilB***

(A) KorB molecules bind as a dimer to the *kilB* operator sequence. Oligomerization of KorB occurs on the DNA (B) shrouding the *kilB* promoter from -40 to +21 (Ducote and Pettis 2003) and largely, if not completely, represses transcription from the *kilB* promoter (Schully and Pettis 2003, Chapter 3 of this study). (C) The RNA polymerase elongation complex transcribing the transmission operon reads through *orf66* (Schully and Pettis 2003) and into the intergenic region separating *orf66* from the *kilB* gene. (D) The RNA polymerase collides with KorB molecules associated with the *kilB* promoter. During the early stages of development, oligomerized KorB molecules are an extremely effective roadblock terminating transcription efficiently at a discrete point (Figure 3.2 B; designated with an asterisk here). This may occur by causing the polymerase to slide backward following the collision, and in so doing, the 3'-end of the elongating transcript is removed from the active site of RNA polymerase (Mosrin-Huaman et al. 2004), followed by release off the transcript (vonHippel and Yager 1992). (E) In the later stages of development, termination is not as efficient and RNA polymerase is allowed to proceed further downstream of the template. This inefficient termination could be the result of modification of the KorB molecule reducing its ability to function as a roadblock. Modification of KorB may reduce its ability to withstand the collision with RNA polymerase, or its ability to oligomerize, thereby reducing the KorB footprint. Either way, transcription proceeds, (F) exposing the IR in the nascent transcript, and (G) allowing formation of the antiterminator hairpin. (H) Formation of the antiterminator hairpin recruits host factors which (I) stabilize the elongation complex allowing (J) readthrough transcription to occur (Nodwell and Greenblatt 1993, Lazinski et al.) through the KorB roadblock (Renan 1989). Following clearance of the promoter by readthrough transcription, RNA polymerase is allowed to (K) initiate transcription from the *kilB* promoter and (L) express *kilB* on a separate transcript (Schully and Pettis 2003).

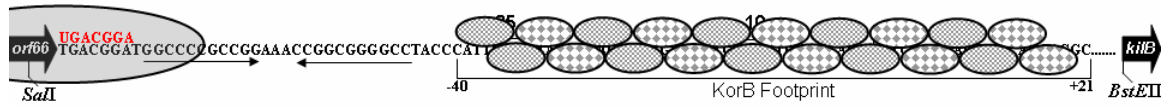
A.



B.



C.



D.

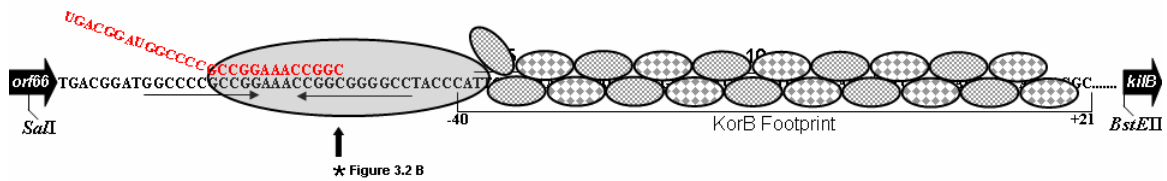
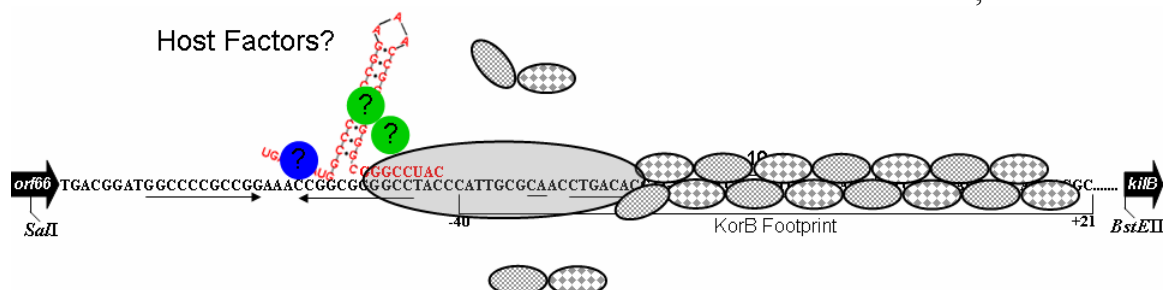


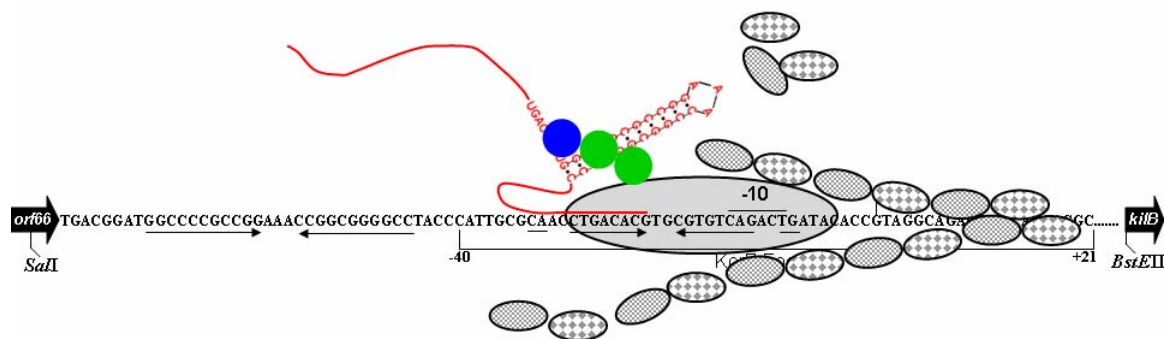
Figure 5.1. (continued)



H.



I.



J.

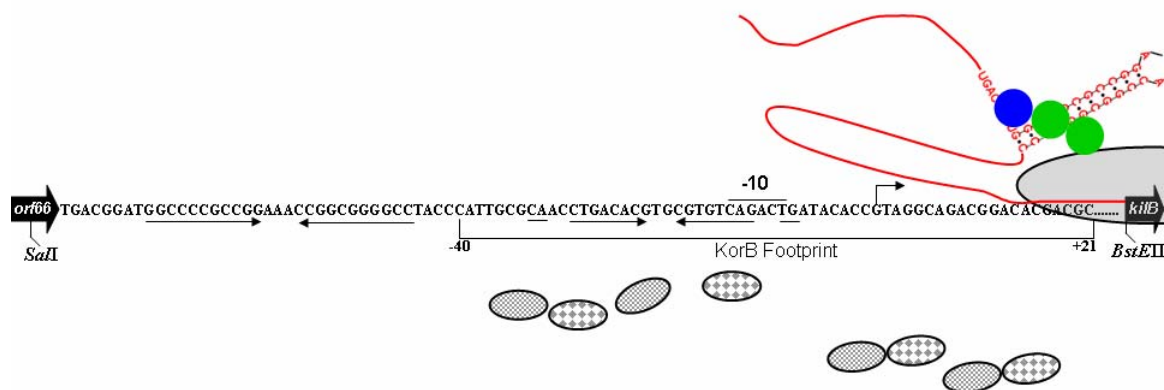
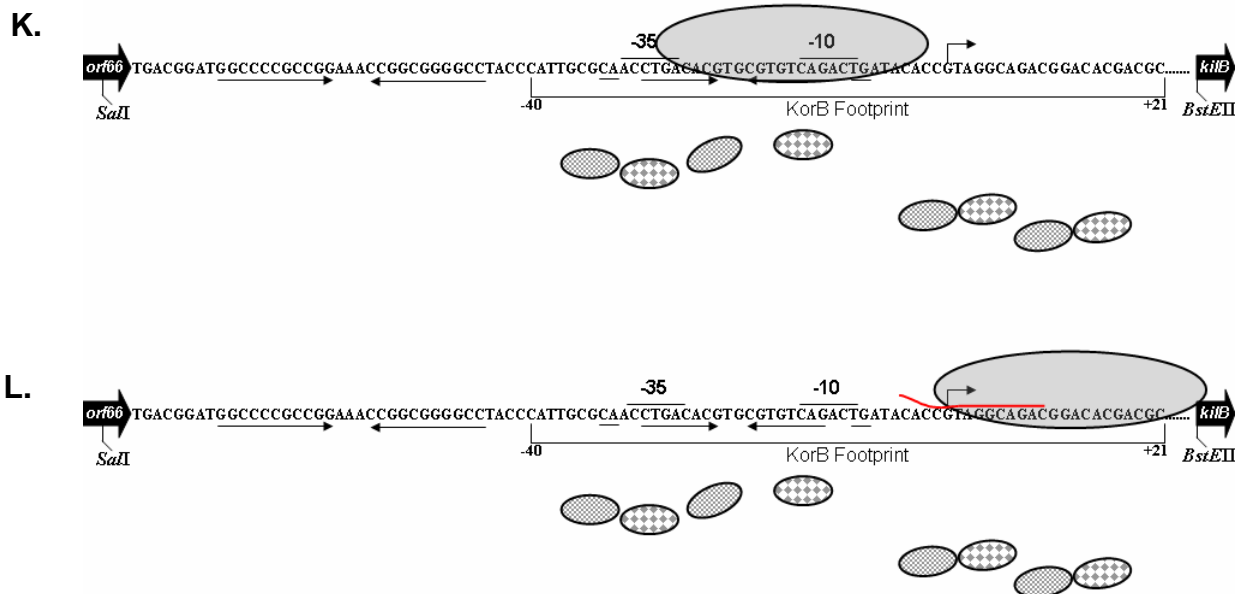


Figure 5.1. (continued)





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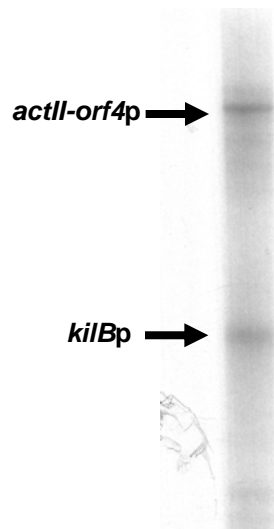
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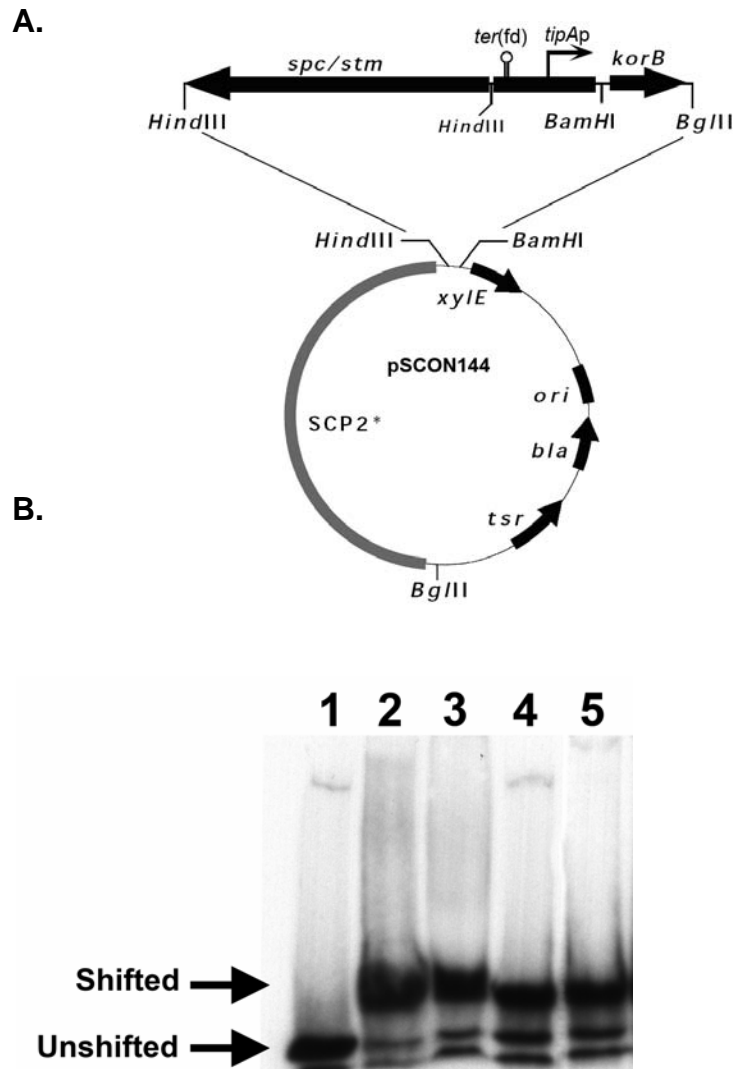
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**APPENDIX A:**  
**SUPPLEMENTARY MATERIAL**



**Figure A.1. In vitro runoff transcription.** In vitro runoff transcription was carried out as described in Kieser et al. (2000) using *S. coelicolor* RNA polymerase (see materials and methods) and a 486-bp DNA template containing the *actII-orf4* and *kilB* promoters generated as described in materials and methods. Full-length runoff transcripts 265-nt long indicate those originating from the *actII-orf4* promoter (*actII-orf4p*) while transcripts 150-nt long indicate transcripts originating from the *kilB* promoter (*kilBp*).



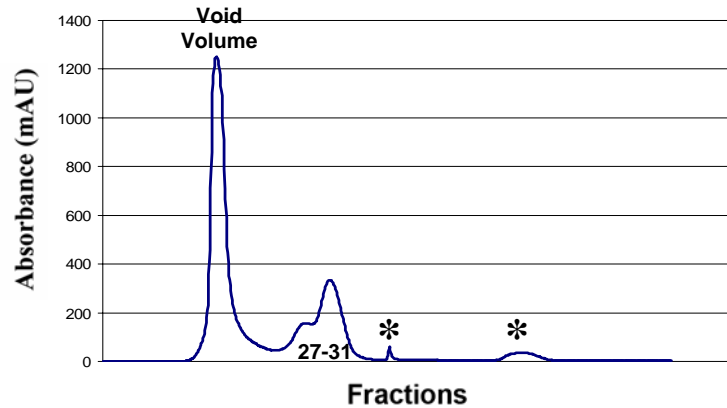
**Figure A.2. Induction and partial purification of KorB**

**A)** pSCON144 was constructed and *korB* expression was induced as described in the materials and methods.

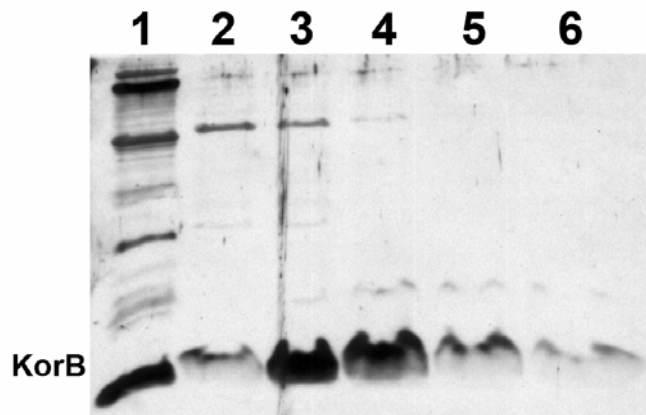
**B)** Electrophoretic mobility shift assay (EMSA) to test KorB production. A 100-bp *EcoRI* fragment containing the *kilB* promoter from pGSP311 was labeled on the ends with  $^{32}\text{P}$  and hybridized with protein extracts as described (Ducote and Pettis 2003).

Crude extracts of *S. lividans* strain 1326 was not capable of shifting of the fragment (lane 1). However crude extracts produced from *S. lividans* strain TK23 harboring pHYG3 (lane 2), and from strain 1326 harboring pSCON144 induced (lane 3), S30 extracts (Kieser et al 2000) of induced cultures harboring pSCON144 (lane 4) and KorB-containing fractions (identified by Western blot) eluted from the heparin column (lane 5) were capable of shifting the *kilB* promoter containing fragment indicating that active KorB protein was present in those extracts or fractions.

**A.**



**B.**

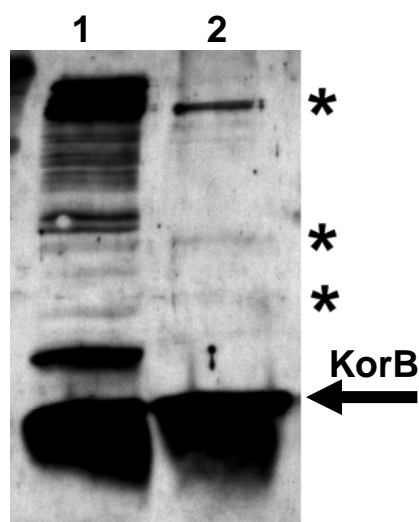


**Figure A. 3. Size exclusion chromatography of KorB-containing heparin fractions.**

**A)** Elution profile of the Sephadex 200 size exclusion column. One milliliter fractions were collected and protein containing fractions were identified by their absorbance at 375 nm (mAU).

**B)** Western blot analysis of gel filtration fractions. Protein-containing fractions were assayed for KorB by Western blot as described (Ducote and Pettis 2003). KorB was found in fractions 27-31.

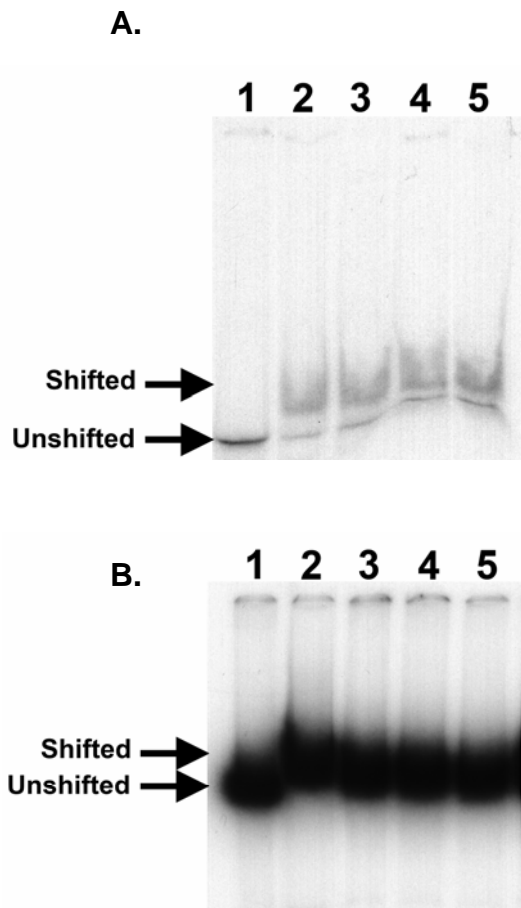
In size exclusion chromatography, larger proteins elute from the column first, followed by the smaller proteins. By this logic, a 6-kDa KorB protein should elute in the later fractions. Interestingly, KorB was identified in the fractions immediately following the void volume (fractions 27-31) and was followed by at least two additional protein-containing peaks (indicated with asterisks). Additionally, the 6-kDa KorB protein eluted with numerous additional proteins that migrated significantly higher than KorB (Figure A.3 B). These results suggest that while in solution, KorB exists in multimeric forms.



**Figure A.4. Evidence for oligomerization of KorB.** Following partial purification by heparin column chromatography, KorB containing fractions were combined and filtered through an Amicon YM10 ultrafiltration device (Millipore) as described in the materials and methods. KorB was then detected in the resulting filtrate by Western blot. Lane 1 contains KorB-containing fractions eluted from a heparin column. Lane 2 contains the YM10 filtrate.

Comparing lanes 1 and 2 demonstrates that filtration dramatically reduced the contaminating proteins and KorB was the predominant protein in the filtered fraction (indicated by an arrow). However, there were also numerous larger bands still present in the filtrate (indicated by asterisks). Because theoretically, the only proteins that can pass through the YM10 are smaller than 10-kDa, the presence of larger proteins indicated by Western blot suggests that KorB is capable of oligomerization. Additionally, it appears KorB has such a high affinity for itself that it remains in multimeric species even when electrophoresed through a denaturing polyacrylamide gel.





**Figure A.5. EMSA analysis of YM10 filtrate.** Concentrated filtrate containing KorB from Figure A.4 was assayed for its ability to bind to the *kilB* promoter. The 100-bp *Eco*RI fragment from pGSP311 (A) and the in vitro transcription template described in Figure A.1 (B) were incubated with crude extracts from *S. lividans* strain 1326 (lane 1) and 1326 harboring pSCON144 induced (lane 2), as well as increasing amounts of the concentrated YM10 filtrate from Figure A.4 (lanes 3-5) and EMSA was otherwise completed as described by Ducote and Pettis (2003).

To determine if the quantity of KorB yielded by the Amicon filtration method was sufficient to completely bind the in vitro transcription template, EMSA was conducted on the 100-bp fragment utilized by Ducote and Pettis (2003) as well as the in vitro transcription template. The quantity of purified KorB was not sufficient to completely shift the 100-bp fragment (A) and did not appear to shift the in vitro transcription template (B) at all. These results indicate that although the KorB produced by the method described in Figure A.4 was active KorB, the quantities yielded were not sufficient to conduct the in vitro transcription experiments described in chapter five.

## Materials and Methods

### In vitro run off transcription

The *actII-orf4* promoter was amplified from *S. coelicolor* genomic DNA using primers *actII-orf4-HindIII*: 5'-AAAAAAGCTTGAGGACCCAGCCGT ATCAG (Kang et al. 1997) and *actII-orf4-SalI* 5'-AAAAGTCGACGGGGGCGACA ATAACAGGC. The resulting 194-bp PCR product was digested with *HindIII* and *SalI* and the 184-bp fragment was cloned into those sites of pSCON201 (Chapter 3), creating pSCON215. The template for in vitro transcription was a 465-bp PCR-amplified fragment from pSCON215, which was created using the *actII-orf4-HindIII* and T7 universal primers. Runoff transcription was performed as described in Kieser et al. (2000) using RNA polymerase isolated from *S. coelicolor* and methods described elsewhere (Achberger and Whiteley 1981, Spiegelman et al. 1978), except that RNA polymerase was eluted from the DNA cellulose using 1 M NaCl. Reaction products were resolved on a 6% polyacrylamide gel containing 8M urea.

### Induction and Purification of KorB

pSCON144 was constructed by PCR amplifying *korB* from pHYG3 using primers *korB5'* - *BamHI*: 5'-AAAAAGGATCCGAAGGAGACTTCAT**G**ACGCAAAGACAC and *korB3'* - *BglII*: 5'-AAAAAGATCTTTGGATCTAGCTTCCGGAGCC and PFU polymerase. Because *korB* does not contain a Shine-Dalgarno sequence, the ribosomal binding site from *korA* was added to *korB5'* - *BamHI* primer (underlined) 5-bp prior to the translation start site (in bold). The resulting 275-bp PCR product was digested with *BamHI* and *BglII* producing a 264-bp product, which was ligated to pSCON111 (M. Ducote, Ph.D. dissertation, Louisiana State University).

*korB* expression was induced by inoculating liquid YEME containing 40  $\mu\text{g ml}^{-1}$  spectinomycin with spores of *S. lividans* strain 1326 harboring pSCON144. Cultures were incubated at 37°C while shaking. When visible growth became evident, the culture was diluted into one liter of YEME containing 40  $\mu\text{g ml}^{-1}$  spectinomycin and 5  $\mu\text{g ml}^{-1}$  thiostrepton, and the cultures were incubated for an additional 48 hrs. Mycelia were harvested by centrifugation and S30 protein extracts were prepared as described in Kieser et al. (2000). Extracts of induced cultures of *S. lividans* strain 1326 harboring pSCON144 were partially purified on a heparin column as described in Tai and Cohen (1993). KorB-containing fractions were identified by Western blot (Ducote and Pettis 2003), and KorB activity was verified by the electrophoretic mobility shift assay (EMSA) as described by Ducote and Pettis (2003).

#### **Gel filtration of KorB**

For size exclusion chromatography, KorB-containing fractions (as determined by Western blot) eluted from the heparin column were combined and dialyzed against TGED (Kieser et al. 2000) for 24h at 4°C and concentrated using a YM3 ultrafiltration device (Millipore). The resulting concentrated fraction was applied to a Sephadex 200 size exclusion chromatography column (Amersham Biosciences), and FPLC was conducted using an ÄKTA (Amersham Biosciences). One milliliter aliquots of column eluate were collected, and KorB containing fractions were identified by Western blot as described (Ducote and Pettis 2003).

#### **Ultrafiltration of KorB**

To filter KorB, extracts of induced cultures of *S. lividans* strain 1326 harboring pSCON144 were partially purified on a heparin column as described in Tai and Cohen

(1993). KorB-containing fractions were combined and incubated in 8M urea at 37°C for 30 minutes. The denatured KorB solution was then forced through an Amicon YM10 (10 kDa molecular weight cut off) ultrafiltration device according the manufacturer's instructions (Millipore), and urea was dialyzed away against increasing concentrations of glycerol (10%, 20% and 30%) at 4°C for a period of ~36 h. The dialyzed fraction was concentrated in a YM3 (3 kDa molecular weight cut off) ultrafiltration device, and KorB was detected by Western blot as described by Ducote and Pettis (2003).

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## **APPENDIX B:**

### **ALTERNATIVE METHOD FOR RNA ISOLATION FROM *STREPTOMYCES* SPECIES ONLY APPROPRIATE FOR CULTURES GROWN SUBMERGED IN LIQUID MEDIA**

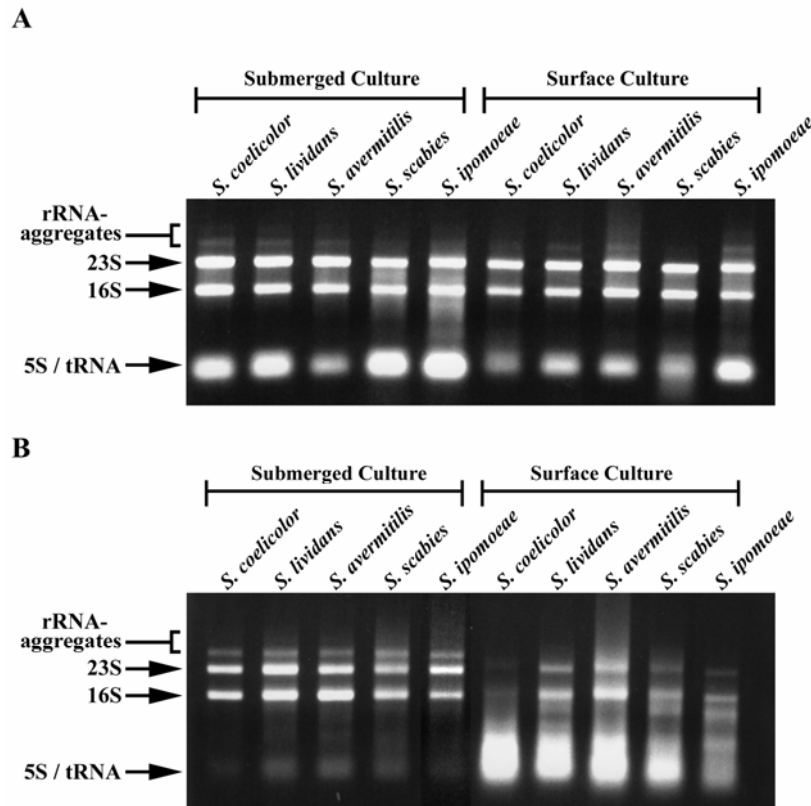
*Streptomyces* bacteria are Gram-positive soil organisms known for their production of a vast array of important natural compounds as well as for a complex life style consisting of three distinct morphological stages. On solid media, *Streptomyces* exists first as a filamentous mass that penetrates the substratum, which is then followed by erection of vertically directed aerial hyphae that eventually produce dispersible spores. However, while growing in submerged culture, species instead typically grow exclusively as bundles of entwined mycelia, though production of secondary metabolites still occurs (Chater 1993). The morphological and physiological complexity of *Streptomyces* has long attracted investigators to studies of its gene expression.

Although reliable, current methods for isolating total RNA from *Streptomyces* species are lengthy cumbersome procedures which typically involve several organic extractions and subsequent multiple treatments with DNase or cesium chloride gradient centrifugation (Kieser et al. 2000). Here, we examined the use of TRI Reagent (Molecular Research Center, Cincinnati, OH), a mixture containing phenol and guanidine thiocyanate, which in a procedure of less than 2 h total duration and involving only a single extraction step is capable of more rapidly isolating RNA that is theoretically free of DNA contamination from a variety of cell types (<http://www.mrcgene.com/rna.htm#Description%20of%20TRI%20Reagent%20>). We found that TRI Reagent yielded RNA from submerged mycelial streptomycete cultures that was of a quality equivalent to that obtained using a traditional isolation procedure; extraction with TRI reagent, however, yielded RNA of very poor quality from the same species throughout their morphological development when growing on agar media.

TRI Reagent was evaluated for its effectiveness in the isolation of RNA from various species of the genus *Streptomyces* including *S. coelicolor* and *S. lividans*, the two most widely used species for genetic studies, *S. avermitilis*, the most recently sequenced species (Ikeda et al. 2003) and producer of the pesticide avermectin, and two plant pathogenic species, the potato pathogen *S. scabies* and the sweet potato pathogen *S. ipomoeae*. For submerged cultures, mycelia were typically grown in yeast extract-malt extract (YEME) media (Kieser et al. 2000) until mid-exponential phase, and following centrifugation RNA was extracted using either TRI reagent as described previously (Schully and Pettis 2003) or a traditional method involving modified Kirby mix for cell lysis with subsequent phenol/chloroform extraction and DNase I treatment (Kieser et al. 2000). For surface cultures, mycelia typically grown in liquid R5 medium (Kieser et al. 2000) until exponential phase were harvested, washed in 0.3M sucrose, and plated onto solid R5 media with cellophane as previously described (Possoz et al. 2001). Unless otherwise indicated, surface cultures were grown for approximately 24 hours, and approximately equivalent amounts of mycelia were collected for the two extraction methods. RNA integrity was evaluated using its ratio of absorbance at 260 nm to 280 nm (with 1.90- 2.05 being considered free of protein contaminants) (Kieser et al. 2000), along with visual assessment by gel electrophoresis (Kieser et al. 2000). All samples were isolated in triplicate with nearly identical results in each case.

As expected, RNA purified using the traditional isolation scheme was of consistently good quality for each of the five species examined regardless of the culture method used (i.e., broth versus agar) (Figure 1A). The average  $A_{260/280}$  ratio of isolated RNA samples was  $1.78 \pm 0.08$  with the range being from 1.68- 1.91. Likewise using the

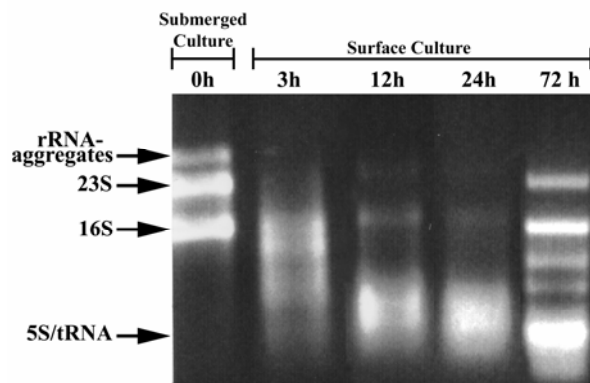
more efficient TRI Reagent method, RNA isolated from broth cultures of each of the five species (Figure 1B, lanes 1-5) was of equivalent quality (i.e., average  $A_{260/280} = 1.80 \pm 0.09$ , with the range being from 1.71-1.95) to that isolated using the traditional scheme. In contrast, however, RNA isolated by TRI Reagent from 24 hr surface cultures of the five species appeared to be severely degraded (Figure 1B, lanes 6-10). Most significantly, the 16S band when visible was always more intense than the degradatively prone 23S, and multiple extraneous lower molecular weight RNA species were apparent for each purified sample.



**Figure B.1. Comparison of RNA isolated from five *Streptomyces* species using traditional versus TRI Reagent extraction methods.** Agarose gel profiles of RNA isolated by either (A) a traditional method involving modified Kirby mixture followed by organic extraction or (B) TRI Reagent extraction. The various *Streptomyces* species indicated were cultivated in liquid or on solid media as described in the text. The positions of rRNA aggregates, 23S rRNA, 16S rRNA and 5S rRNA/tRNA are indicated.



To compare the quality of RNA isolated using TRI Reagent from different morphological stages of surface growth, mycelia from broth cultures of *S. coelicolor* were plated as before but then following growth for varying durations cells were harvested and then immediately lysed in TRI Reagent. Interestingly, as shown in Figure 2, cells growing apparently as a substrate mycelium after only 3 h of surface growth yielded RNA of poor quality using TRI Reagent, while, as expected, cells collected immediately upon plating (0 h) yielded highly intact RNA. RNA isolated at subsequent times following plating including 12 h, which was later during substrate mycelial growth, 24 h, which was when aerial hyphae were first evident, and at 72 h following sporulation was also of poor quality. Unlike mycelia from broth cultures, surface-grown cells never appeared to lyse as efficiently in TRI Reagent; although the exact basis for this inefficient lysis remains undetermined, the quality of RNA from surface cultures did not improve when attempts were made to scale up the extraction procedure such as by increasing the ratio of TRI Reagent volume to mass of cells harvested (data not shown).



**Figure B.2. Effects of surface growth phase and duration on integrity of RNA isolated using TRI Reagent.** From an exponentially growing broth culture of *S. coelicolor*, mycelia were plated and at that point (0 h) or at subsequent times representative of *Streptomyces* surface growth (i.e., 3 h and 12 h, early and late substrate mycelium respectively; 24 h, aerial hyphae; 72 h, spores) RNA was isolated from the corresponding cell types using TRI reagent.

We therefore conclude that TRI reagent is inappropriate for use with morphologically differentiating mycelia and traditional, tried and true methods should be utilized. However, TRI Reagent is an attractive alternative for the isolation of RNA from *Streptomyces* bacteria grown exclusively in liquid culture. RNA purified by this method was found to be suitable for numerous sensitive assays (e.g., microarray and quantitative PCR analyses), and though the procedure involved only one organic extraction, less DNA contamination was evident than with the traditional method (data not shown). Finally, while only relatively small scale isolations were attempted here, it should be noted that starting with 1.5 ml of an exponentially growing culture that had an absorbance at 600 nm equal of approximately 0.8 an average of 25 µg of total RNA was isolated, a yield that should prove suitable for a variety of molecular biological analyses.

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**APPENDIX C:**  
**LETTERS OF PERMISSION**



23 February 2005

Our ref: HG/HDN/FEB05/J171

Mr Kevin Schully  
Louisiana State University  
Dept of Biological Sciences  
107 Life Sciences Blvd  
Baton Rouge, LA 70803  
USA

Dear Mr Schully

**JOURNAL OF MOLECULAR BIOLOGY, Vol 334, No 5, 2003, pp 875 – 884, K L Schully et al, “Separate...”**

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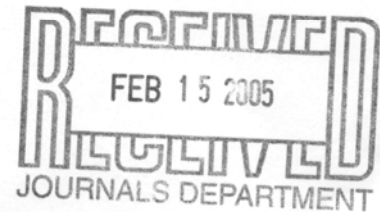
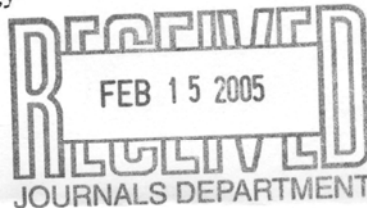
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February 14, 2005

Linda Illig  
American Society for Microbiology  
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I am writing to request permission to reproduce two figures from a Journal of Bacteriology article on which I am the third author in my doctoral dissertation. The article citation is: Pettis, G.S., N. Ward, and K.L. Schully. 2001. Expression characteristics of the transfer-related *kilB* gene product of *Streptomyces* plasmid pIJ101: implications for the plasmid spread function. J. Bacteriol. 183: 1339-1345. I would like to incorporate figure 1 with minor modifications and figure 3 exactly as it appears in the article.

I can be contacted with the information below.

Sincerely,

Kevin Schully  
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107 Life Sciences Bld  
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Date 2-16-05

## **VITA**

Kevin Lee Schully was born on May 21, 1973, in New Orleans, Louisiana, he was the third child of his parents Lloyd and Sylvia Schully. He was raised with his older brother and sister, Gary and Eileen, in Kenner, Louisiana and attended Archbishop Rummel High School in Metairie, Louisiana. Kevin attended Louisiana State University where he graduated with a Bachelor of Science degree in 1999. That same year, Kevin entered the graduate program under the guidance of Dr. Gregg S. Pettis in the Department of Biological Sciences. While working toward his doctorate, Kevin married Sheri Dixon, also a graduate student in the Department of Biological Sciences. Kevin and Sheri will both earn the degree of Doctor of Philosophy in biological sciences and will graduate in August of 2005.